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理學博士學位論文

*Streptomyces coelicolor*에서 산화환원 센서인 SoxR의
기능과 조절

Regulation and Function of Redox Sensor SoxR in
Streptomyces coelicolor

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**Regulation and Function of Redox Sensor
SoxR in *Streptomyces coelicolor***

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Abstract

Redox-sensitive transcription factor SoxR in enteric bacteria regulates cellular response toward superoxide and nitric oxide via inducing the expression of a downstream regulator SoxS, that activates more than 100 genes. In other bacterial groups, however, SoxR directly induces its multiple target genes in response to redox-active compounds, as initially demonstrated for pseudomonads. The antibiotic-producing soil bacterium *Streptomyces coelicolor* contains a gene for SoxR homologue (SCO1697) whose DNA-recognition helix is identical to that of *Escherichia coli* SoxR. Using *E. coli* SoxR binding sequence, five candidate genes of SoxR regulon were predicted. It was demonstrated that SoxR binds to their promoter regions and activates their expression concurrently with the production of blue polyketide antibiotic actinorhodin (a benzoisochromanequinone). These genes encode probable NADPH-dependent flavin reductase (SCO2478), NADPH-dependent quinone reductase (SCO4266), ABC-transporter (SCO7008), monooxygenase (SCO1909), and a hypothetical protein (SCO1178). Addition of actinorhodin to exponentially growing cells activated the expression of SoxR target genes in a SoxR-dependent manner.

SoxR from *E. coli* and related enterobacteria is activated by a broad range of redox-active compounds through oxidation or nitrosylation of its [2Fe-2S] cluster. In contrast, non-enteric SoxRs appear to get activated by a narrower range of redox-active compounds that include endogenously produced metabolites. The responsiveness of SoxRs from *Streptomyces coelicolor* (ScSoxR), *Pseudomonas aeruginosa* (PaSoxR) and *E. coli* (EcSoxR), all expressed in *S. coelicolor*, were compared toward natural or synthetic redox-active

compounds. EcSoxR responded to all compounds examined, whereas ScSoxR was insensitive to oxidants such as paraquat (Eh -440 mV) and menadione sodium bisulfite (Eh -45 mV) and to nitric oxide (NO) generators. PaSoxR was insensitive only to some NO generators. Whole cell EPR analysis of SoxRs expressed in *E. coli* revealed that the $[2\text{Fe-2S}]^{1+}$ of ScSoxR was not oxidizable by paraquat, differing from EcSoxR and PaSoxR. The mid-point redox potential of purified ScSoxR was determined to be -185 ± 10 mV, higher by ~ 100 mV than those of EcSoxR and PaSoxR, coinciding with its insensitivity to paraquat. The overall sensitivity profile indicates that both redox potential and kinetic reactivity determine the differential responses of SoxRs toward various oxidants. Residues within the $[2\text{Fe-2S}]$ binding site, which are specific to ScSoxR, were mutated and were evaluated for their effects on the sensitivity profile.

Key Words; *Streptomyces coelicolor*, SoxR, Fe-S, redox-active compounds, EPR, redox potential, oxidative stress, superoxide

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Abbreviations

aa	amino acids
bp	base pairs
nt	nucleotide
MD	menadione
MDs	menadione sodium bisulfite
PQ	paraquat (Methyl viologen)
PL	plumbagin,
PMS	phenazine methosulfate
Pyo	Pyocyanin
Tox	Toxoflavin
SNP	sodium nitroprusside
GSNO	S-nitrosoglutathione
DETA-NO	diethylenetriamine/nitric oxide adduct
RACs	reactive active compounds
Cys	cysteine
kDa	kilo Dalton
DTT	dithiothreitol
PAGE	polyacrylamide gel electrophoresis
ONPG	Ortho-nitrophenyl-b-D-galactopyranoside
IPTG	isopropyl-b-D-thiogalactopyranoside
Amp	ampicillin
Kan	kanamycin
Apr	apramycin
EPR	Electro paramagnetic resonance spectroscopy

CHAPTER I

INTRODUCTION

I.1. Biology of *Streptomyces coelicolor*

Streptomyces are the most widely studied and well-known genus of the actinomycete family and ubiquitous Gram-positive soil bacteria with a unique capacity for the production of varied and complex secondary metabolites. They are crucial in soil environment because of their broad range of metabolic processes. They usually inhabit soil and are important decomposers. They are crucial in soil because of their broad range of metabolic processes and biotransformations. These include degradation of the insoluble remains of other organisms, such as lignocellulose and chitin, making *Streptomyces* central organisms in carbon recycling (McCarthy & Williams, 1992). The importance of *Streptomyces* to medicine results from their production of over two-thirds of naturally derived antibiotics in current use (Bentley *et al.*, 2002; Lucas *et al.*, 2013).

Unusually for bacteria, *Streptomyces* undergo complex multicellular developmental life cycle. *Streptomyces* life cycle starts from germination of spore and formation of highly branched vegetative mycelium. The hyphae are divided into multigenomic compartments by the infrequent formation of vegetative septa. After a period of active growth, aerial mycelium develops from substrate mycelium on the surface of colony, and eventually differentiates into unigenomic spores (Fig.I-1). Genetic studies on morphological differentiation started from the isolation of mutants with altered morphology: bld (bald) mutants, which fail in aerial mycelium formation, and whi mutants which are defective in sporulation (Merrick, 1976; Potůčková *et al.*, 1995; Pope *et al.*, 1998; Kelemen *et al.*, 1996; Nodwell *et al.*, 1996; Flärdh & Buttner, 2009). The morphological differentiation is often temporally associated with physiological differentiation, the production of secondary metabolites, as synthesis generally occurs after the main period of rapid growth and assimilative metabolism (Chater, 1984; Demain, 1983;

Matsumoto *et al.*, 1995; Onaka *et al.*, 1998).

Streptomyces species have been the subject of genetic investigation for over 50 years, with many studies focusing on the developmental cycle and the production of secondary metabolites. Among them, *Streptomyces coelicolor* is genetically the best-known representative of the genus. The complete DNA sequence of *S. coelicolor* M145 has been published recently, with others expected to follow soon (Bentley *et al.*, 2002). The linear chromosome is 8,667,507 bp long and is predicted to contain 7,825 genes, about twice as many as typical free-living bacteria, making it the largest bacterial genome yet sequenced. The genome shows a strong emphasis on regulation, with 965 proteins (12.3%) predicted to have regulatory function. This is not only attractive feature but also for a challenging puzzle for future investigation to elucidate gene regulation in this organism.

I.2. Oxidative stress responses

All living organisms have developed adaptive systems to cope with environmental changes during growth and maintain cellular homeostasis. Changes in growth condition may occur either as a natural consequence of cellular growth or from abrupt changes in environmental conditions such as nutrient, temperature, osmolarity, pH, and redox state.

Reactive oxygen species (ROS) are produced as an inescapable consequence of aerobic life or by exposure to radiation, stimulated macrophages, or redox-active drugs (Fig. I-2). Because of their high reactivity, the oxidants can damage cells in many ways: by inactivating proteins, damaging nucleic acids, and altering the fatty acids of lipid, which leads in turn to perturbations in membrane structure and function. The accumulation of this oxidative damage underlies the formation of many disease states in humans (Graves, 2012). It is suggested that tissue injury by these ROS

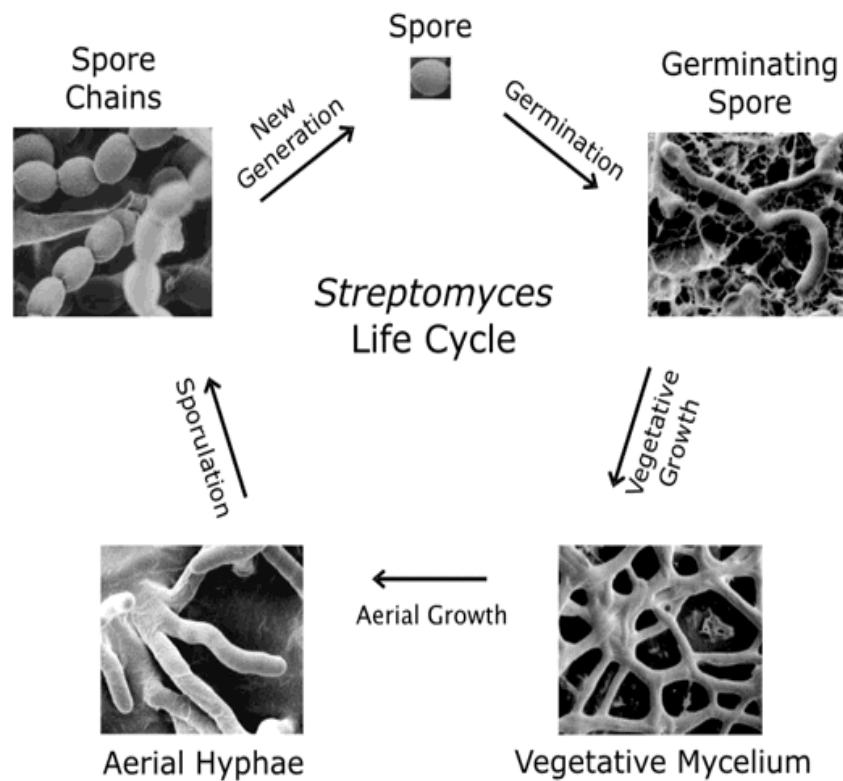


Fig. I-1. The life cycle of *S. coelicolor*

From a single spore a vegetative mycelium germinates, this is followed by aerial growth with the production of aerial hyphae. These hyphae in turn will undergo synchronous septation to produce unigenomic spore compartments, which will disperse and thus commence a new cycle. This figure is adopted from (McGregor, 1954)

accumulates over age and plays roles in the aging process and the development of heart disease, diabetes, chronic inflammatory disease, cancer, and several neurodegenerative disease (Halliwell & Gutteridge, 1991). In order to encounter oxidative damage, cells have evolved antioxidant defense systems including both the constitutive and adaptive responses (Moyano *et al.*, 2014).

I.3. Reactive oxygen species

Molecular oxygen itself is a rarity, a stable diradical, with two spin-aligned, unpaired electrons in its π antibonding orbitals. An important consequence of this structure is that organic molecules with spin-paired electrons cannot transfer more than one electron at a time to oxygen. Because oxygen is a relatively weak univalent electron acceptor and most organic molecules are poor univalent electron donors, this restriction ensures that oxygen cannot efficiently oxidize amino acids and nucleic acids. However, the unpaired electrons of dioxygen readily interact with the unpaired electrons of transition metals and organic radicals (Imlay, 2003; Peet, 2012).

Its reactivity increases upon serial one electron reduction of oxygen, or a spin flip to become singlet oxygen ($^1\Sigma_gO_2$). The resulting oxygen derivatives, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\bullet$), and singlet oxygen are collectively defined as reactive oxygen species (ROS).

However, the anionic charge of O_2^- inhibits its effectiveness as an oxidant of electron-rich molecules, while the reactivity of H_2O_2 is diminished by the stability of its oxygen-oxygen bond. Neither of these features applies to the hydroxyl radical, and indeed $HO\bullet$ reacts at virtually diffusion-limited rates with most biomolecules.

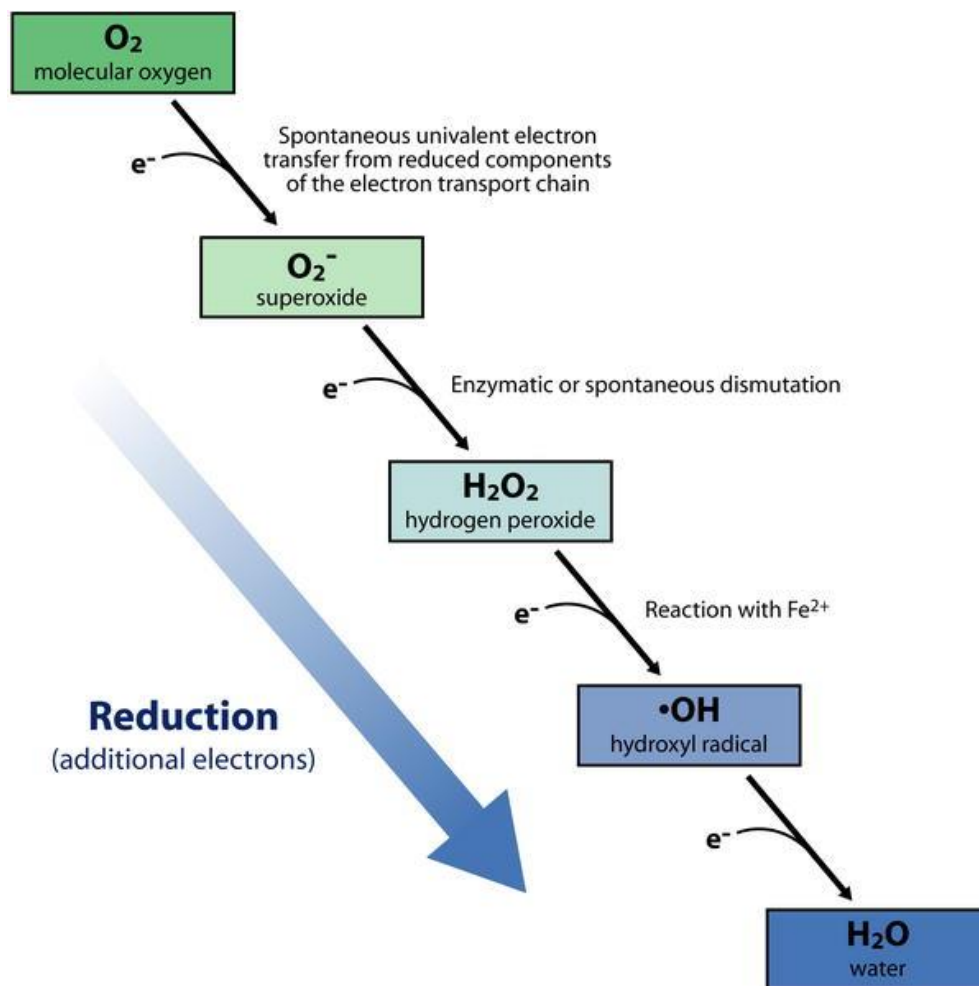


Fig. I-2. Formation of Reactive Oxygen Species

The four-electron reduction of molecular O_2 generates two molecules of H_2O , which is O_2 in its most reduced form. While this reduction normally occurs within the enzyme cytochrome oxidase, one-electron transfers to O_2 also occur outside of cytochrome oxidase via inadvertent reactions with other reduced electron carriers, resulting in partially reduced and reactive forms of O_2 . H_2O_2 is also produced by the enzymatic or spontaneous dismutation of O_2^- , and $\bullet OH$ is generated by the reaction of iron with H_2O_2 (the Fenton reaction). In addition, the reactive oxygen intermediates are produced by a variety of organisms as a defense against microbial invasion. This figure is adopted from (Kiley & Storz, 2004)

Superoxide radical (O_2^-)

The superoxide radical is generated by one electron transfer to one of the two orbitals of the ground state of oxygen. In *Escherichia coli*, autooxidation of membrane-associated respiratory chain enzymes, such as NADH dehydrogenase and succinate dehydrogenase are major sources of O_2^- generation in vivo (Messner & Imlay, 2002; Korshunov & Imlay, 2010). Nonenzymatic production of O_2^- occurs by oxidation of several cellular components including ubiquinols, thiols, and flavins (Massey *et al.*, 1969; Simonian & Coyle, 1996). Electrophilic quinone compounds, both natural and cellular constituents (ubiquinone and plastoquinone) and exogenous sources (plumbagin and menadione), are an important group of substrate for flavoproteins catalyzing either two electron reduction to the hydroquinone or one electron reduction to the semiquinone radical (Bellomo *et al.*, 1990). Semiquinones readily reduce O_2 to O_2^- , regenerating the oxidized quinones. The oxidized quinones can repeat this cycle and it thus referred to as a redox-cycling agent. Superoxide radical can oxidize thiols, ascorbate, tocopherol, and catecholamines (Fridovich, 1989; Powers & Jackson, 2008). The major target of O_2^- damage identified in bacteria is a class of dehydratase enzymes that utilize [4Fe-4S] clusters to bind their substrate (Imlay, 2003). O_2^- is dismutated to H_2O_2 and O_2 , spontaneously or enzymatically by superoxide dismutase (SOD)(Sharma *et al.*, 2012).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is generated by dismutation of O_2^- and by autooxidation of flavoenzymes such as sulfite reductase and xanthine oxidase (Imlay, 2003; Messner & Imlay, 2002). Some carbon sources autooxidize and thereby contribute to H_2O_2 formation (Seaver & Imlay, 2001). It can act as weak oxidizing agent and oxidize cysteinyl residues; creating sulfenic acid adducts that can either form disulfide cross-links with other cysteines or be

oxidized further to sulfinic acid moieties (Kiley & Storz, 2004). It can also oxidize methionine residues to methionine sulfoxide and a variety of carbonyls (Griffiths & Cooney, 2002). However, its more significant action is to generate more reactive OH^\bullet radical by reaction with reduced iron or copper ions by the Fenton reaction (Cadenas, 1989; Lemire *et al.*, 2013). Since O_2^- can elicit both H_2O_2 production and reduction of Fe^{3+} and Cu^{3+} , it also enhances HO^\bullet generation.

Hydroxyl radical (HO^\bullet)

The only oxygen species that can directly damage most biomolecules is HO^\bullet . It is formed when ferrous iron transfers an electron to H_2O_2 (Fenton reaction). The hydroxyl radical oxidizes most organic molecules (RNA, DNA, protein, and lipid) at diffusion-limited rates because HO^\bullet has high reactivity due to its very high standard electrode potential (Singh & Singh, 1983; Flora, 2009). While the Fenton reaction has been linked to protein carbonylation and membrane peroxidation, its most significant impact is likely to be upon DNA, since even a single DNA lesion is potentially mutagenic or lethal.

Singlet oxygen ($^1\Sigma\gamma\text{O}_2$)

Singlet oxygen may be generated from hydroxyl radical by the action of certain enzymes, such as decomposition of superoxide or peroxidized glutathione, and photosensitization reactions with endogenous sensitizers such as riboflavin and bile pigments (Foote, 1982; Cabiscol *et al.*, 2010). It can be formed by energy transfer to O_2 by excited chromophores and is generated in photosynthetic system. Almost all reactions involving singlet oxygen with biomolecules are addition of the $^1\text{O}_2$ to conjugated bond. The known targets are carotenes, chlorophylls, and fatty acid side chains present in the lipid membrane, suggesting that $^1\text{O}_2$ can initiate lipid peroxidation (Kappus, 1985).

I.4. Redox active compounds

Bacteria are exposed to a variety of redox-active molecules that include reactive oxygen and nitrogen species as well as organic compounds. Some of these agents are generated endogenously, whereas bacteria encounter others in their external environment. Plants and bacteria produce a variety of redox-active metabolites, some of which we identify as quorum signals, virulence factors, antibiotics, toxins, etc., depending on the life phenomena that we are interested in (Okegbe *et al.*, 2012). When internalized in the bacterial cytoplasm, some of these redox-active compounds can generate superoxide anion radicals by abstracting electrons from redox enzymes and then transferring them to O₂. This cycle is catalytic, thus befitting the name 'redox-cycling' agent.

The redox-active compounds are recognized that these compounds are released by both plants and bacteria as devices to inhibit the growth of competitors (Inbaraj & Chignell, 2004). For example, plumbagin, a naphthoquinone, was originally isolated from the plant *Plumbago*; juglone, another quinone, occurs naturally in the *Juglandaceae* family and is recognizable as the yellow residue on the leaves and seeds of the black walnut (Inbaraj & Chignell, 2004). Both compounds are effective herbicides that allow the parent plant to dominate a habitat. Phenazines are commonly excreted by bacteria, including *Pseudomonas*, *Streptomyces* and *Pantoea agglomerans* (Turner & Messenger, 1986).

Pyocyanin, an endogenous redox-active antibiotic produced by *Pseudomonas aeruginosa*, activates transcription of two genes/operons encoding a probable efflux pump and a monooxygenase that might aid in phenazine transport and modification (Dietrich *et al.*, 2006).

Streptomyces produce a variety of secondary metabolites, including antibiotics. The best-characterized model organism *S. coelicolor* produces two

pigmented antibiotics called actinorhodin and undecylprodigiosin (Chater, 1993). Actinorhodin, a benzo-isochromanequinone, belongs to a class of aromatic polyketides and resembles the phenazine ring structure (Okamoto *et al.*, 2009). It is a pH indicator that turns from red to blue at neutral to alkaline pH and hence is called a “blue” antibiotic (Brockmann & Hieronymus, 1955). Its synthetic enzymes are encoded from a gene cluster (act genes, SCO5076 to SCO5092) (Bentley *et al.*, 2002) and are composed of a polyketide synthase complex that produce a 16-carbon polyketide backbone and a variety of modifying enzymes to produce the six-ringed actinorhodin molecules that accumulate in the cell. Intracellular actinorhodin is converted to a lactone form called γ -actinorhodin during or after export from the cell (Bystrykh *et al.*, 1996). Actinorhodin export is mediated through an efflux pump, ActA, whose expression is regulated by a TetR-like repressor, ActR, in response to the production of actinorhodin (Bystrykh *et al.*, 1996; Tahlan *et al.*, 2007).

I.5. Mechanisms of oxidative cell damage

O_2^- and H_2O_2 have different chemical reactivities and generate distinct types of damage inside cells. Mutants of *E. coli* that lack cytosolic superoxide dismutase cannot grow in air without amino acid supplements, cannot catabolize non-fermentable carbon sources, and exhibit high rates of spontaneous mutations (Castro *et al.*, 1994; Keyer & Imlay, 1997). Most of these phenotypes have been traced to a single type of injury, the oxidative inactivation of a family of dehydratases. These enzymes utilize exposed iron-sulfur clusters [4Fe-4S] to bind and dehydrate substrates; dehydratase oxidation by O_2^- provokes cluster disintegration and a loss of enzyme activity (Flint *et al.*, 1993; Jang & Imlay, 2007). The auxotrophy of superoxide dismutase mutants for branched-chain amino acids and their inability to catabolize non-fermentable carbon sources reflect the inactivation of

dihydroxyacid dehydratase and of aconitase and fumarase, respectively (Fridovich, 1995). A by-product of iron-sulfur cluster damage is that copious iron is released into the cytosol, where it catalyzes the oxidation of DNA in conjunction with H_2O_2 (Liochev & Fridovich, 1994).

I.5.1. Biological defense systems to oxidative stress

Oxidative stress has been defined as a disturbance in the prooxidant-antioxidant balance in favor of pro-oxidants (Sies, 1994). Thus, conditions that lead to increased level of reactive oxygen species (ROS) or conditions that lead to the depletion of antioxidant molecules or enzymes constitute an oxidative stress (Uttara *et al.*, 2009). For aerobically growing bacterial cells, the autooxidation of components of the respiratory chain is the main sources of endogenous O_2 and H_2O_2 (González-Flecha & Demple, 1995; Imlay & Fridovich, 1991). Increased levels of ROS are also caused by exposure to radiation, metals, and redox-active drugs (Christophersen, 2012). In addition, plants, microorganisms, and animals all possess mechanisms to specifically generate oxidants as a defense against bacterial invasion. The ROS are deleterious to cells since they can lead to protein, DNA, and membrane damage (Kishikawa *et al.*, 2012). Genes encoding antioxidant enzymes can be detected in the sequence of most completed genomes, showing that defenses against oxidative stress are critical to many organisms.

Prevention of generation of ROS can be achieved by chelating metal ions such as iron and copper, which promote generation of free radicals. Metal binding proteins (ferritin, transferrin, and metallothionein etc.) and transition metal-containing enzymes (cytochrome oxidase, CuZn superoxide dismutase etc.) are responsible for this first line defense. Furthermore, nonspecific DNA-binding protein (Dps) revealed to be homologous to ferritin, suggesting that Dps may also protect against DNA damage by sequestering

iron (Grant *et al.*, 1998; Nair & Finkel, 2004; Nguyen & Grove, 2012). Both enzymatic and non-enzymatic defense systems are involved in auto-oxidant defense by scavenging ROS. Catalase, peroxidases, and superoxide dismutases (SOD) constitute the major enzymatic defense system. Catalase decomposes H_2O_2 to O_2 and H_2O , while peroxidase uses intracellular reductants to reduce H_2O_2 (Loew, 1901; Miller *et al.*, 2000). Peroxidases have been classified by the kinds of electron donors: glutathione peroxidase, NADH peroxidase, ascorbic acid peroxidase, and bromo (chloro) peroxidase (Halliwell & Gutteridge, 1991). Catalase peroxidase, which has been identified only in prokaryotes, have both catalytic and peroxidase activities. SOD catalyzes the dismutation of superoxide to O_2 and H_2O_2 . Superoxide dismutases (SOD) can be classified based on the metal ions present at their active site: Mn-SOD, CuZn-SOD, Ni-SOD, and Fe-SOD (Fridovich, 1998).

Non-enzymatic ROS scavengers include α -tocopherol (vitamin E), ascorbic acid (vitamin C), β -carotene, and uric acid, which have diverse antioxidant function (Beyer, 1994). Molecules such as glutathione and glutaredoxin, and thioredoxin reduce disulfide bond caused by oxidative effect in protein. Glucose 6-phosphate dehydrogenase (zwf), which is a metabolic enzyme, contributes to defense against oxidative stress by producing reducing power, NADPH (Giró *et al.*, 2006).

Repair system exists as a final safe guard against oxidative damage. Damaged DNA can be repaired by enzymes such apurinic/apyrimidine (AP) endonuclease, DNA glycosylase, and exonuclease III. Similarly, damaged proteins can be protected by chaperones or removed by proteolysis (Tell *et al.*, 2009; Slade & Radman, 2011). Recently, Hsp33 was identified as a member of newly discovered family of heat shock proteins, whose chaperone activity was induced by disulfide bond formation with concomitant release of coordinating zinc (Jakob *et al.*, 1999). The lipid peroxide can be repaired by

alkyl-hydroperoxide (Ahp) reductase system. In animals, phagocytes employ NADPH oxidase, nitric oxide synthase, and myeloperoxidase to bombard captured bacteria with O_2^- , NO^- , $HOCl$, and their chemical by-products, H_2O_2 , HO , $HOONO$, and $RSNO$. Although these enzymes contribute to the killing of bacteria in vivo, it is not yet clear which products are directly responsible for toxicity. Unlike the other chemicals, O_2^- ($pK_a = 4.8$) cannot cross membranes at neutral pH. It may, however, conceivably do so in the acidic pH of the phagolysosome. Each of these reactive oxygen and nitrogen species is bacteriostatic or bacteriocidal in vitro, but their impacts in vivo will depend upon their ultimate concentrations, currently unknown, inside the captured bacterium.

I.5.2. SoxR and the SoxRS response to superoxide stress in *E. coli*

The cell has different responses to O_2^- versus H_2O_2 . The main regulator of H_2O_2 induced oxidative stress is OxyR regulon; OxyR is a transcriptional activator that senses H_2O_2 levels in the cell by the formation of a disulfide bridge between two of its cysteine residues (Lee *et al.*, 2004). However, constitutive induction of this OxyR is not sufficient to protect cells against redox cycling agents that produce O_2^- . The two oxidants have been shown to induce different DNA damage responses, and unlike H_2O_2 , O_2^- induced stresses do not trigger the SOS response. The protein expression profiles are also different when comparing the two stressors. However, some differences in protein expression were observed when comparing the protein expression profiles of *E. coli* lacking any superoxide dismutase (SOD) activity and wild type *E. coli* treated with the redox cycling agents paraquat and plumbagin (Walkup & Kogoma, 1989).

The SoxRS response, which protects against superoxide-generating

agents and nitric oxide (NO), is triggered by activation of a sensor molecule, SoxR, containing two essential [2Fe-2S] clusters. The SoxRS regulon is induced in a two-stage process. Upon activation, SoxR induces *soxS* expression and SoxS, in turn, activates transcription of genes of the regulon. The mechanism of signaling has been under debate for years (Fig. 1.3.). Evidence for several pathways of SoxR activation mediated by the modification of [2Fe-2S] centers, has emerged from recent data. The direct oxidation of [2Fe-2S] centers, any event that may interfere with the pathway maintaining SoxR in a reduced inactive form, direct nitrosylation by NO can trigger SoxR activation. The multiple possibilities for SoxR activation, along with signal amplification via the two-stage process, constitute a unique, and particularly sensitive to a broad range of environmental changes indicative of possible oxidative stress. Recently, SoxR homologue was found in *Salmonella typhimurium*. The *S. enterica* Serovar Typhimurium SoxRS system also mediates redox-inducible resistance to diverse antibiotics, which may be relevant to clinical infections. The SoxR protein is expressed constitutively and is a homodimeric transcriptional regulator that contains redox-active iron-sulfur clusters (Hidalgo *et al.*, 1995; Wu *et al.*, 1995). Genome-wide mutational analysis was used to identify the regulon responsible for the unique O_2^- response in *E. coli* (Greenberg *et al.*, 1990; Tsaneva & Weiss, 1990). This regulon, named SoxRS (superoxide response) is comprised of two genes: *soxR*, which encodes a 17 kDa, 154-amino acid transcription factor which operates as a homodimer, and its target gene *soxS*, which encodes as 13 kDa transcription factor (Wu & Weiss, 1991). SoxR operates from a single site on the genome, at the *soxS* promoter containing a palindromic recognition element and allosterically downregulates its own expression by acting as a repressor at its own promoter site. This down regulation results in a low copy number of SoxR in the cell (<100 nM) (Hidalgo *et al.*, 1998). The promoter

region is characterized by an 18-base pair palindromic recognition element, and the footprint of SoxR on the promoter region stretches 36-base pairs long. SoxR is a member of the MerR family of transcription factors, which includes ZntR, and CueR, which senses Zn(II) and Cu(II) respectively. These proteins share a common homology; they all function as homodimers, and each subunit contains a C-terminal metal-binding domain that is specific for a unique metal, a dimerization interface, and an N-terminal helix-turn-helix DNA binding domain (Amabile-Cuevas & Demple, 1991). The mode of action is also conserved; these proteins bind in the region of the promoter usually bound by repressors, and the spacing between the -10 and -35 conserved boxes in the promoters of their gene targets is longer than the 18-base pair consensus spacing optimal for RNA polymerase binding (Fig. I.3).

Upon metal binding, the protein induces a large conformational change in the DNA, which allows RNA polymerase to bind and transcribe the target gene. However, unlike the other members of the MerR family, SoxR contains a redox-active [2Fe-2S] cluster in place of a metal or drug-binding domain, which is crucial for its activity, but is not important structurally. There is one cluster per monomer of SoxR, ligated by four cysteine residues (Cys-119, Cys-122, Cys-124, and Cys-130)(Gaudu *et al.*, 1997).

This cluster is known to exist in two oxidation states, the reduced [2Fe-2S]⁺ form and the oxidized [2Fe-2S]²⁺ form. Reduced SoxR is EPR active, and has signals with g-values of 2.01, 1.92, and 1.90; oxidized SoxR is EPR silent.

In cells, the clusters of SoxR are maintained in a reduced [2Fe-2S]⁺ state (Ding *et al.*, 1996), and a one electron oxidation of a cluster to the oxidized [2Fe-2S]²⁺ state allows SoxR to function as a transcriptional activator (Ding *et al.*, 1996; Hidalgo & Demple, 1996; Hidalgo & Demple, 1997). Importantly, the cluster is not critical for DNA binding, and apo-SoxR binds

DNA with comparable affinity to the intact protein ($K_d = 4.5 \times 10^{-10}$ M) both in its reduced and oxidized forms (Hidalgo & Demple, 1994; Wu *et al.*, 1995). Thus, SoxR remains bound to the promoter region of its target gene in its inactive state. The binding site and footprint region of SoxR on the *soxS* promoter is shown in Fig. I.4.

Two models have been proposed for the activation of SoxR. The first model states that it is regulated through the reversible assemble/disassemble of [2Fe-2S] center, either partial or complete. Disassembly may be facilitated by thiol. It cannot easily explain why SoxR that was purified from uninduced cells contained inactive Fe-S centers, unless one assumes that there is efficient spontaneous reconstitution of Fe-S cluster in cell extracts. The alternative model states that SoxR is regulated by the univalent oxidation of its Fe-S cluster, which remains intact. SoxR was readily auto-oxidized, and the protein that is isolated in ambient atmosphere from uninduced cells is transcriptionally active. SoxR can be reversibly inactivated *in vitro* by reduction of its Fe-S centers with dithionite under condition that should not have resulted in dissociation of Fe-S centers. This finding was consistent with an observation that the regulon's induction is sensitive to redox balance of the cells. Recently, it has been reported that mutations mapped to two loci. The *rsxABCDGE* operon (named for reducer of SoxR) that is highly homologous to the *rmfABCDGE* operon in *R. capsulatus* involved in transferring electrons to nitrogenase, and the *rseC* gene in the *rpoE-rseABC* operon may be a reducing system for SoxR (Koo *et al.*, 2003).

SoxR acts as a transcriptional activator by changing the conformation of DNA at its binding site, as illustrated in Fig. I.4. The *soxS* promoter region contains a 19-base pair spacing between the conserved promoter boxes, which corresponds to a 6.8 Å increase in translational length and a rotation of 72° around the axis compared to the consensus 17-base pair spacing. SoxR

itself is not thought to interact with RNA polymerase directly; instead, the elongated spacer regions described here preclude binding of RNA polymerase and initiation of transcription even in the absence of SoxR (Hidalgo & Demple, 1997). A recently solved crystal structure of the oxidized form of SoxR bound to DNA shows that the transcriptionally active form of the protein induces a 65° bend, as shown in Fig. I.4., and partial unwinding of the DNA at the promoter site, which results in a shortening of the length of the region by 3.4 Å, or 1 base pair. This change enables RNA polymerase to bind and initiate transcription (Watanabe *et al.*, 2008).

The SoxR/SoxS paradigm is only true for enterics. Though SoxR analogues have also been identified in bacteria, which lack SoxS, in these species, SoxR binds to multiple sites on the genome and directly activates a variety of genes with different functions. Furthermore, the role of SoxR can diverge from that of responding to elevated levels of O₂⁻ in the cell. In these organisms, agents other than O₂⁻ are able to activate SoxR, and in some cases, deletion of the *soxR* gene has no effect on O₂⁻ resistance (Dietrich *et al.*, 2008).

Nitric oxide (NO) has diverse roles in intercellular communication and (at higher levels) in immune-mediated cell killing. NO reacts with many cellular targets, with cell-killing effects correlated to inactivation of key enzymes through nitrosylation of their iron-sulfur centers. SoxR protein is also activated in *E. coli* on exposure to macrophage-generated NO⁻. SoxR activation by NO occurs through direct modification of the [2Fe-2S] centers to form protein-bound dinitrosyl-iron-dithiol adducts, both in intact bacterial cells and in purified SoxR after NO⁻ treatment. Nitrosylated SoxR has transcriptional activity similar to that of oxidized SoxR and is relatively stable. In contrast, nitrosylated SoxR is short-lived in intact cells, indicative of mechanisms that actively dispose of nitrosylated iron-sulfur centers.

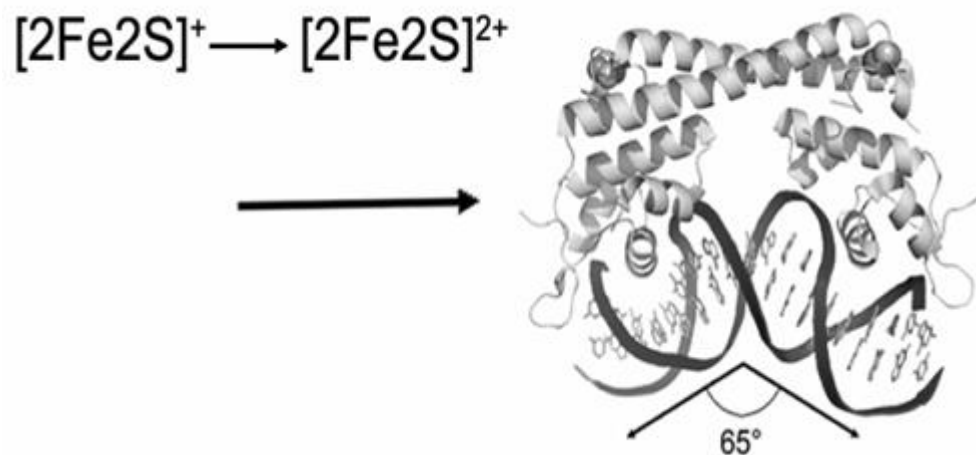


Fig. I.4. The transcriptional activity of SoxR is mediated by its [Fe-S] clusters. The one-electron oxidation of one of the [2Fe-2S] clusters from the +1 state to the +2 state induces a conformational change in the DNA at the promoter site of *soxS*, the target gene for SoxR. The protein induces a 65° bend in the DNA, which amounts to a ~1 base pair shortening of the length of the region between the -10 and -35 promoter elements. This shortening of the spacer region allows RNA polymerase to bind to the promoter and transcribe *soxS*. This figure is adopted from (Lee, 2009).

It is well established that SoxR is activated in the presence of redox cycling drugs that form the reactive oxygen species O_2^- but the exact nature of SoxR oxidation is also not known. In fact, SoxR has been reported to be activated by H_2O_2 and NO^\bullet as well (Nunoshiba *et al.*, 1993). However, the low copy number of this protein in the cell and the fact that, it diffusible species is responsible for oxidizing the iron-sulfur clusters of SoxR. Furthermore, O_2^- is likely to irreversibly degrade the cluster of SoxR upon direct interaction. An alternate hypothesis is that these redox-cycling agents interfere with the ability of the cell to maintain SoxR in a reduced state by consuming reducing equivalents to form O_2^- from O_2 ; the same scenario might arise in cells undergoing oxidative stress. Common cellular redox binds to a single site on the genome with high affinity makes it unlikely that a buffers such as glutathione do not reduce SoxR, nor do redox cofactors such as NADPH or NADH.

Recently, a possible reducing system for SoxR was identified as the *rsxABCDGE* operon, which shares homology with the *rnf* operon in *Rhodobacter capsulatus* involved in nitrogen fixation; the gene products of this operon have not been well characterized and a direct interaction with SoxR has not been demonstrated (Koo *et al.*, 2003).

I.5.3. *Salmonella typhimurium* SoxR

SoxR homologue was also found in *Salmonella typhimurium*. The *S. enterica* Serovar Typhimurium SoxRS system mediates redox-inducible resistance to diverse antibiotics, which may be relevant to clinical infections. They responds to superoxide-generating agents through soxR-mediated activation of the *soxS* gene, whose product, SoxS, is necessary for resistance to oxidative stress (Pomposiello and Demple 2001). This system was used to demonstrate that *soxS* expression is sufficient for the induction of resistance to the superoxide-generating drug paraquat as well as NO generating compound (Koutsolioutsou *et al.*, 2001) and for the transcriptional activation of the *sodA* and *micF* genes.

I.5.4. *Pseudomonas* SoxR

In *Pseudomonas aeruginosa* SoxR has an open reading frame (ORF) encoding a putative protein homologous to *E. coli* SoxR, but not to SoxS. Instead of a *soxS* homolog, ORFs encoding an unknown hypothetical protein and *soxR* are arranged divergently with their 5' ends separated by a 78 bp region containing a sequence homologous to the SoxR-binding *soxS* promoter. (Kobayashi & Tagawa, 2004; Palma *et al.*, 2005). SoxR activated by endogenous redox-active antibiotic pyocyanin, a signaling molecule with pleiotropic functions, directly targets a limited number of genes, none of which encodes superoxide dismutase and the genes are putative transporters and monooxygenase that can modify substrates through hydroxylation (Dietrich *et al.*, 2006; Dietrich *et al.*, 2008). In Fig. I.5. shown responding to several synthetic redox-cycling drugs, the SoxR regulon genes in several of these organisms are induced by endogenously produced redox-active metabolites, including pyocyanin in *Pseudomonas* species and there regulation mechanism are shown in Fig. I.6. (Dietrich *et al.*, 2008). The SoxR protein

functions as an autorepressor in the absence of paraquat, whereas in the presence of paraquat, this autorepression is diminished (Ha & Jin, 1999). Evidence indicating that *P. aeruginosa* SoxR activates a six-gene regulon in response to O₂⁻ induced stress. The regulon includes three transcriptional units: (i) the recently identified *mexGHI-ompD* four-gene operon, which encodes a multidrug efflux pump system involved in quorum-sensing signal homeostasis; (ii) gene PA3718, encoding a probable efflux pump; and (iii) gene PA2274, encoding a probable monooxygenase. Demonstrate that *P. aeruginosa* SoxR is not a key regulatory player in the oxidative stress response. Finally, it is shown that the *E. coli*-based SoxRS paradigm does not hold in *P. aeruginosa* and fosters a new hypothesis for possible physiological role of *P. aeruginosa* SoxR. (Palma *et al.*, 2005).

In case of *P. putida*, genome did not reveal a clear *soxS* homolog. *P. putida* SoxR protein appears to be functional: its expression well in an *E. coli* *soxR* strain restored the paraquat inducibility of *soxS*. There are nine candidate *P. putida* oxidative stress genes, which are known to be SoxR regulon in *E. coli*, tested for response to superoxide or nitric oxide, *fumC-1*, *sodA*, *zwf*⁻¹ and particularly *fpr*, encoding ferredoxin:NADP⁺ reductase, were induced, all independent of *P. putida* *soxR*. However, *P. putida*Δ*soxR* had normal resistance to the O₂⁻ generating agent PQ. It is shown that the genetic responses to O₂⁻ stress in *P. putida* differs from those seen in *E. coli* and *Salmonella*, and the role of *P. putida* *soxR* remains to be established (Park *et al.*, 2006) .

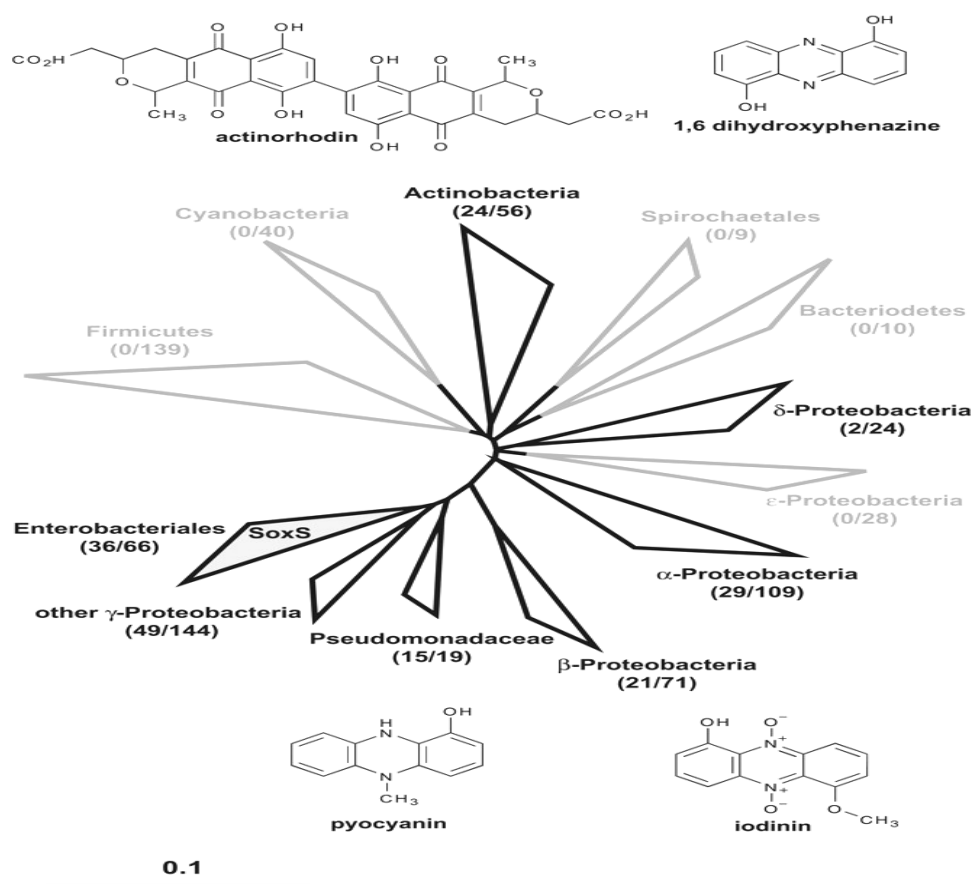


Fig. I.5. Distribution of SoxR and SoxS among phyla of the domain Bacteria.

A BLAST search for *E. coli* SoxR and SoxS was performed, and SoxS was found only in enterics. SoxR homologs were identified in 176 α , β , δ , γ -Proteobacteria and Actinobacteria. All of these homologs contain the SoxR-specific cysteine motif CI[G/Q]CGC[L/M][S/L]XXXC required for binding of the [2Fe-2S] cluster. The number of hits within respective phyla are indicated, followed by the total number of genomes surveyed. Members of these phyla (in black) are noted for their ability to produce and excrete redox-active small molecules, such as phenazines (Turner & Messenger, 1986) and actinorhodin (Chater, 2006). Representative structures are shown. The tree was constructed using the ARB neighbor joining method from 16S ribosomal RNAs of 604 bacterial species. This figure is adopted from (Dietrich *et al.*, 2008).

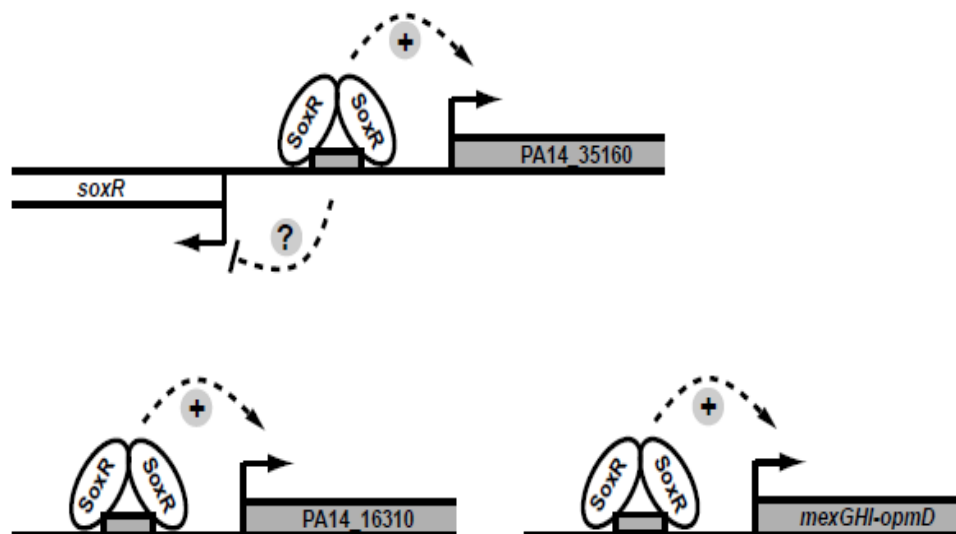


Fig. I.6. Organization of SoxR regulons in *P. aeruginosa*. The *P. aeruginosa* SoxR regulon differs from the *E. coli* paradigm. In *E. coli*, the SoxR homodimer binds to the soxRbox in the soxS promoter region. soxR and soxS are divergently transcribed. The binding of reduced SoxR to the soxRbox represses expression of soxR and soxS. Oxidation of the SoxR [2Fe-2S] cluster induces a conformational change that allows transcription of soxS. SoxS regulates genes involved in superoxide tolerance and detoxification. In contrast, in *P. aeruginosa*, the gene adjacent to soxR encodes a putative monooxygenase. Two additional soxR boxes, found elsewhere in the *P. aeruginosa* genome, regulate expression of putative drug transporters. This figure is adopted from (Lee, 2009).

I.5.5. *Xanthomonas campestris* SoxR

In *Xanthomonas campestris* pv. *campestris*, SoxR likely functions as a sensor of redox-cycling drugs and as a transcriptional regulator. Oxidized SoxR binds directly to its target site and activates the expression of xcc0300, a gene that has protective roles against the toxicity of redox-cycling compounds. In addition, SoxR acts as a noninducible repressor of its own expression. *X. campestris* pv. *campestris* requires SoxR both for protection against redox-cycling drugs and for full virulence on a host plant. The *X. campestris* model of the gene regulation and physiological roles of SoxR represents a novel variant of existing bacterial SoxR models (Mahaviahakanont *et al.*, 2012).

I.5.6. *S. coelicolor* SoxR

In *Streptomyces coelicolor*, it has been proposed that two predicted SoxR regulon genes encoding putative oxidoreductases (SCO2478, SCO4266) are expressed in cells that are capable of producing pigmented antibiotics but not in non-producing cells (Dietrich *et al.*, 2006). Considering that many of the SoxR regulon-containing bacteria also produce redox-active antibiotics (Turner & Messenger, 1986), it has been hypothesized that SoxR may regulate transport and turnover of small redox-active molecules (Dietrich *et al.*, 2006). Later, it was reported that *S. coelicolor*, SoxR (ScSoxR) is activated by endogenous actinorhodin, a polyketide antibiotic, and induces genes for two putative NADPH-dependent reductases (SCO2478, SCO4266), an ABC transporter (SCO7008), a monooxygenase (SCO1909), and a hypothetical protein (SCO1178) (Dela Cruz *et al.*, 2010; Shin *et al.*, 2011)(Table 1). All known target genes of SoxR, whether from *E. coli*, *P. aeruginosa*, or *S. coelicolor*, share similar binding sequence for SoxR, consistent with the conservation of DNA-binding residues in SoxR.

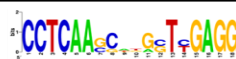
Interestingly, the *soxS* gene is confined to enterobacteria, whereas

soxR is found in a wide range of bacteria such as proteobacteria (α , β , γ , δ), and actinobacteria (Dietrich *et al.*, 2008). Furthermore, studies in non-enterics suggested that in these organisms SoxR has a different physiological impact than in enterics. In pseudomonads (Palma *et al.*, 2005; Park *et al.*, 2006), *Agrobacterium tumefaciens* (Eiamphungporn *et al.*, 2006), *Xanthomonas campestris* (Mahavithakanont *et al.*, 2012), and *S. coelicolor* (Dela Cruz *et al.*, 2010; Shin *et al.*, 2011) SoxR directly targets a limited number of genes, none of which encodes superoxide dismutase. Furthermore, in addition to responding to several synthetic redox-cycling drugs.

The SoxR regulon genes in several of these organisms are also induced by endogenously produced redox-active metabolites, including pyocyanin in *Pseudomonas* species (Dietrich *et al.*, 2008) and actinorhodin in *S. coelicolor* (Dietrich *et al.*, 2008; Dela Cruz *et al.*, 2010; Shin *et al.*, 2011). These departures from the *E. coli* paradigm led to a reconsideration of the generalized function of SoxR and of the mechanism of its activation. Contrary to a long-held idea of SoxR activation by superoxide, a recent work put forward the idea that SoxR is primarily activated by redox-active metabolites, not by superoxide, even in *E. coli* (Gu & Imlay, 2011). This was based on such observations that SoxR can be activated in vivo under anoxic conditions in the absence of any superoxide and that the [2Fe-2S] of purified SoxR can be directly oxidized by redox-cycling agents in vitro (Gu & Imlay, 2011). Superoxide may be able to activate SoxR (Liochev & Fridovich, 2011; Fujikawa *et al.*, 2012). However, probably with too low an efficiency to act as a physiological signal (Gu & Imlay, 2011). The anoxic activation of SoxR by changes in the intracellular NADPH/NADP⁺ ratio (and possibly NADH/NAD⁺) supports this idea as well (Krapp *et al.*, 2011). Therefore, a generalized mechanism of SoxR activation seems to exist across bacterial phyla (Dietrich & Kiley, 2011).

Table I.1. Genes with SoxR-binding sequence in *S. coelicolor*

Function	No. SCO	Mismatch	Predicted SoxR binding sequences
<i>E.coli soxSp</i>		0	<u>TTTA</u> <u>CCTCAAGTTAACTTGAGG</u> AAT <u>TATACT</u>
Putative reductase	SCO2478	2	<u>TTGA</u> <u>CCTCAAGCAAACTTGAGG</u> TAC <u>CAGGCT</u>
Putative oxidoreductase	SCO4266	4	<u>TTGA</u> <u>CCTCAAGCAGGCTTGAGG</u> TCG <u>TTGAGT</u>
Putative ABC type transporter	SCO7008	5	<u>TTGA</u> <u>CCTCAAGGTGGTTCGAGG</u> TTC <u>TACGGT</u>
Unknown	SCO1909	6	<u>TTGA</u> <u>CCTCAACCTTGGTTCGAGG</u> TCG <u>GAGGGT</u>
Unknown	SCO1178	7	<u>TTGA</u> <u>CCTCAACCGTGGTTCGAGG</u> TAC <u>CACGGT</u>



Arrows on top of *E. coli* SoxS sequence indicate the 18 bp inverted repeat (bold) we used as a query to screen putative SoxR target genes by using RSAT program (<http://rsat.bigre.ulb.ac.be/rsat/>). The predicted -10 and -35 promoter elements with 19 nt spacing were underlined. The number of nucleotides that deviated from the *E. coli* SoxS promoter sequence was shown (mismatch) and the diverged sequences were presented in non-bold italic. -n- indicates the distance between -10 and translational start codon (**ATG or GTG**). This table is adopted from (Shin *et al.*, 2011).

Even though the DNA-binding property of SoxR is conserved, the oxidation or activation behavior seems quite different among different SoxRs, in terms of responsiveness (selectivity) toward a range of chemicals. A recent work proposed that PaSoxR and ScSoxR respond to a narrower range of chemicals than does EcSoxR (Sheplock *et al.*, 2013). These non-enteric SoxRs were reported to be less sensitive to low-potential viologens such as paraquat (<-350 mV), leading to the hypothesis that PaSoxR and ScSoxR share structural properties that delimits which chemical signals are effective (Sheplock *et al.*, 2013). Mutagenesis identified residues that were essential for the ability of EcSoxR to respond to paraquat (Chander *et al.*, 2003). Some of these residues differ in PaSoxR and ScSoxR, and the “non-enteric type” residues were mutagenized to the “enteric” type in an effort to pinpoint the mechanistic/structural determinants of selectivity. This analysis implicated three “non-enteric” residues in restricting the sensitivity in PaSoxR toward paraquat (Chander *et al.*, 2003).

I.6. Aims of this study

S. coelicolor is the model species for the study of many fundamental genetic phenomena in *Streptomyces*. Its unique character, the complex life cycle during growth, made it a good model system to study the relationship between oxidative stress, metal homeostasis and differentiation. In this study, we examined the activation behavior and function of SoxR in *S. coelicolor*. We also compared the sensitivity profiles of three representative SoxRs toward a range of redox-active compounds by expressing all proteins in *S. coelicolor*, thereby circumventing problems that might arise from the differential permeability of compounds into their native organisms. Our results demonstrate that of the three SoxRs, ScSoxR is the most limited in the range of chemicals to which it responds and has the highest reduction potential. It does serve to protect cells against the growth-inhibiting effect of inducing chemicals. Both kinetic and equilibrium (redox potential) factors determine the range of effective chemicals.

CHAPTER II

MATERIALS AND METHODS

II.1. Bacterial strains and culture conditions

II.1.1. *Streptomyces coelicolor*

S. coelicolor A3(2) M145 was used as wild type in most studies. *Streptomyces* cells were grown as described previously (Kieser *et al.*, 2000). For liquid culture, spore suspension was inoculated in YEME medium (1% glucose, 0.5% Bacto Peptone, 0.3% malt extract, 0.3% yeast extract, 10.3% sucrose, 5 mM MgCl₂), Nutrient Broth (NB, 0.4% beef extract, 0.4% beef peptone; commercially supplied as Nutrient Broth by Biolife) media were used. NMMP (0.2% (NH₄)₂SO₄, 0.5% Difco casaminoacids, 0.06% MgSO₄·7H₂O, 5% PEG 6000, 15 mM NaH₂PO₄/K₂HPO₄ (pH 6.8), 5% glucose), or NMMP chelated with Chelex-100 resin (Bio-Rad) for 2 hrs before addition of phosphate buffer and glucose and cells were grown at 30 °C with vigorous shaking. For surface culture, R5 (10.3% sucrose, 1.1% MgCl₂·6H₂O, 1% glucose, 0.5% yeast extract, 0.6% TES, 0.01% cas-aminoacids, 0.025% K₂SO₄, 2.2% agar; after autoclave 0.3% L-proline, 0.007N NaOH, 20 mM CaCl₂·2H₂O, 0.005% KH₂PO₄), R2YE (10.3% sucrose, 1% glucose, 1% MgCl₂, 0.024% K₂SO₄, 0.001% cas-aminoacid (Difco), 0.5% yeast extract, 20 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.0), 20 mM CaCl₂, 0.005% K₂HPO₄, and 0.3% proline), NA (nutrient agar plate; 8% nutrient broth, 2.2% agar), and SFM (soy flour mannitol plate; 2% soy flour, 2% mannitol, 2% agar) were used. To facilitate harvesting of mycelia, inoculums were spread on cellophane membrane on solid media. To apply oxidative stress in liquid culture, various concentrations of oxidants was treated to exponentially growing cell (OD₆₀₀ = 0.3~0.5).

II.1.2. *Escherichia coli*

Escherichia coli strain DH5 was routinely used for manipulation of DNA. For overexpression of recombinant proteins using T7 polymerase-based system, *E.coli* BL21 (DE3) pLysS was used according to the

manufacturer's recommendations (Novagen). To gain methylation-negative DNA, *E. coli* ET12567 (Macneil *et al.*, 1992) was used, and for direct transformation of *S. coelicolor*, DNA was introduced into *E. coli* ET12567 harboring pUZ8002 (lab collection) to supply the donor transformation when having a compatible oriT-containing plasmid. *E. coli* BW25113 (Datsenko & Wanner, 2000) was used to propagate the recombination plasmid pIJ790 and *S. coelicolor* cosmids (Redenbach *et al.*, 1996). Cells were grown to mid-exponential phase to OD₆₀₀ of 0.3-0.5 for treatment with chemicals and to prepare RNA. *E. coli* cells were grown in LB medium at 37°C. Strains were grown in LB (1% tryptone, 0.5% yeast extract, 1% NaCl) or SOB (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl) containing 20 mM MgSO₄ under aeration at 37°C or 30°C. Carbenicillin (Carb, 100 µg/ml), apramycin (Apr, 50 µg/ml), chloramphenicol (Cm, 25 µg/ml), or kanamycin (Kan, 50 µg/ml) were added to growth media when required. L-arabinose (10 mM final concentration) was added as indicated to SOB medium to induce genes under control of the pBAD promoter (Datsenko & Wanner, 2000). All *E. coli* strain used in this study are listed in Table II-1.

II.1.3. *Pseudomonas aeruginosa*

P. aeruginosa cells were grown aerobically at 37°C. LB medium was used for routine culturing (1% tryptone, 0.5% yeast extract, 1% NaCl). Carbenicillin (Carb, 100 µg/ml), apramycin (Apr, 50 µg/ml), chloramphenicol (Cm, 25 µg/ml), or kanamycin (Kan, 50 µg/ml) were added to growth media when required. *P. aeruginosa* strain used in this study is listed in Table II-2.

Table II-1. *E. coli* strains used in this study.

Strains	Genotype or description^a	Source or reference
DH5α	F ⁻ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>endA1</i> <i>recA1</i> <i>hsdR17</i> <i>deoR</i> <i>supE44</i> <i>thi-1</i> λ ⁻ <i>gyrA96</i> <i>relA1</i>	Hanahan, 1983
BL21(DE3)pLysS	F ⁻ <i>ompT</i> <i>r_B</i> ⁻ <i>m_B</i> ⁻ (DE3)/pLysS	Studier, 1991
ET12567(pUZ8002)	F ⁻ <i>dam13::Tn9</i> <i>dcm6</i> <i>hsdM</i> <i>hsdR</i> <i>recF143::Tn10</i> <i>galK2</i> <i>galT22</i> <i>ara-14</i> <i>lacY1</i> <i>xyl-5</i> <i>leuB6</i> <i>thi-1</i> <i>tonA31</i> <i>rpsL136</i> <i>hisG4</i> <i>tsx-78</i> <i>mtl-1</i> <i>glnV44</i>	McNeil <i>et al.</i> , 1992
BW25113	K12 derivative: <i>araBAD</i> , <i>rhaBAD</i>	Datsenko and Wanner, 2000

II.2. Chemical treatments

γ -actinorhodin (Act) was isolated from a surface culture of *S. coelicolor* M145 cells on R2YE plates as described previously (Shin *et al.*, 2011). The following chemicals were purchased from Sigma: pyocyanine (Pyo), methyl viologen (PQ), phenazine methosulfate (PMS), plumbagin (PL), menadione sodium bisulfite (MDs), menadione (MD), sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), diethylenetriamine/nitric oxide adduct (DETA-NO) and IPTG (Isopropyl β -D-1-thiogalactopyranoside). Toxoflavin (Tox) was kindly provided by Prof. Ingyu Hwang (Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, SNU). Stock solutions were made fresh and were diluted to final indicated concentrations.

II.3. DNA manipulations

Purification of plasmid DNA from *E. coli*, digestion with restriction enzymes, gel electrophoresis, ligation, and transformation of *E. coli* competent cells were carried out as described previously (Sambrook *et al.*, 1989). Purification of genomic or plasmid DNA from *S. coelicolor* were done following the method described by (Kieser *et al.*, 2000).

II.3.2. DNA isolation and purification

DNA isolation, purification, and elution from agarose gel were carried out as described by Sambrook *et al.* (1989). Plasmid DNA was extracted by the alkaline lysis method (Bimboim & Doly, 1979) or plasmid mini prep kit (Takara).

II.3.3. General recombinant DNA techniques

DNA modification reactions, such as DNA digestion, phosphorylation, dephosphorylation, ligation, and DNA polymerization, were carried out as

described by Sambrook *et al.*, (1989) or by the manufacturer's instructions.

II.3.4. DNA sequencing

DNA sequencing was done by automatic sequencer (ALFexpress, Pharmacia). Primers for sequencing were designed to hybridize at the flanking regions of the cloning sites of pUC18, pSET162, pTAC1, pTAC4, and pET vector.

II.3.1. Polymerase Chain Reaction (PCR)

Each 100 μ L reaction mixture contained the followings; 1 \times Taq polymerase reaction buffer, 150 μ M MgCl₂, 200 μ M dNTP, 500 nM each of 5' and 3' primers, 10-100 ng of template DNA, and 5 U of Taq polymerase. Reaction was carried out in thermal cycler (Thermo) by denaturing at 95°C, annealing at different temperature for different samples, and extending at 72°C and cycle is 15-35 cycle.

II.4. PCR-targeted disruption genes in *S. coelicolor*

II.4.1. Construction of Δ *soxR* mutant.

The Δ *soxR* mutant was constructed through PCR-targeted mutagenesis by replacing the entire coding sequence of SCO1697 with the apramycin-resistance cassette [*aac(3)IV*]. The upstream forward primer used to create Δ *soxR* mutation contained the *soxR* gene sequences up to the start codon (bold) linked with *aac(3)IV* sequence (underlined) (5'-GTCCGCGCG GGCGATCGGGTCGGTAGGGTTCGAGGGT**G**ATTCCGGGGATCCGTCGA CC-3'). The downstream reverse primer corresponds to *soxR* stop codon (bold) and its downstream connected with the *aac(3)IV* sequence (underlined) (5'GCCACGACTGACGACCGGGCCAGGGTCACCGCGCGGT**CATGTAGG** CTGGAGCTGCTC-3'). The purified PCR product was introduced by

electroporation into *E. coli* BW25113 (Datsenko & Wanner, 2000) strain that harbors the λ red recombination plasmid pIJ790 and a cosmid SC8F11 (a gift from John Innes Centre) that contains the *soxR* gene. The resulting recombinant cosmid [SC8F11 Δ *soxR::apr*] recovered from the selected transformants was verified for its gene structure and introduced into *E. coli* ET12567 carrying pUZ8002, followed by conjugal transfer to *S. coelicolor* M145. Apramycin-resistant and kanamycin-sensitive exconjugants were selected, and we isolated three colonies that contained the expected gene structure as follows. Genomic PCR using forward primer (5'-GGTGTACCCCAAATGCTCGC-3' for *soxR* upstream position) and reverse primer (5'-CCCGAGGTGCGACGACGGGT-3' in *soxR* coding region) excluded colonies with wild type gene structure, and Southern hybridization of genomic DNA with *aac(3)IV* gene probe for the presence of apramycin cassette. The three Δ *soxR* isolates showed similar phenotypes, and we used one of the isolates for further experiments.

II.4.2. Construction of Δ *soxR* strains expressing ScSoxR, EcSoxR, or PaSoxR

The Δ *soxR* mutant of *S. coelicolor* (Shin *et al.*, 2011) was transformed with pSET162-based recombinant plasmids containing ORFs for ScSoxR, EcSoxR, or PaSoxR. To construct the recombinant plasmids, DNA fragments containing the promoter of the *S. coelicolor* *soxR* gene (*soxRp*) and the coding sequences of the *soxR* genes from *S. coelicolor*, *E. coli*, or *P. aeruginosa* were amplified by PCR and cloned into the pGEM-Teasy plasmid (Promega). The 823, 707 and 715 bp long fragments containing the *soxRp-soxR* region were cut out with *EcoRI* and *BamHI* restriction enzymes and cloned into pSET162, which is a derivative of integration vector pSET152 with a thiostrepton resistance marker (Bierman *et al.*, 1992). The pSET162-based recombinant plasmids were introduced into methylation-negative, conjugal host strain

E. coli ET12567 and then transferred to the $\Delta soxR$ mutant by bacterial conjugation. The proper chromosomal integration in exoconjugants that showed apramycin^R and thioestrepton^R phenotypes was verified by genomic PCR analysis. For expression studies in *E. coli*, the $\Delta soxR$ mutant of GC4468 strain (Table II-2.) was transformed with pTac4-based recombinant plasmids containing ORFs for ScSoxR, EcSoxR, or PaSoxR.

II.4.3. Construction of truncated and Swapped ScSoxR

Sco truncated consist of V1 to R158 of SoxR, for making Sco truncated ORF, Sco truncated -up (5'-GGTTCGAGCATATGCCTCAGATTC-3'; Nde I site underlined) and Sco truncated -down (5'-GCGGCTCGGGATCCGGCC TCACCTGGCGGTG-3'; BamHI site underlined). The 501 bp PCR product was digested with NdeI and BamHI and cloned into pTac4 vector. SWAP1 consist of Sco V1 to S125 and Eco R127 to N154. For this purpose, a overlapping PCR was done. the primer pair ScoSoxR-up (5'-GGT TCGAGCATATGCCTCAGATTC-3'; NdeI site underlined) and ScoSoxR-down (5'-GCAGCCGCAGCCGATGCAGTCGGTGAGGTGGTCGCGCAG-3') were used to create the upstream half, and the pair EcSoxR-up (5'-CTGCCGCGACCACTCACCAGCTGTATTGGTTGTGGCTGCCTT-3') and EcSoxR-down (5'-GCGCCCTGGATCCGCTTTAG-3'; BamHI site underlined) were used to create the downstream half and for SWAP2 consist of Sco V1 to C129 and Eco P131 to N154. the primer pair ScoSoxR-up (5'- GGT TCGAGCATATGCCTCAGATTC-3'; NdeI site underlined) and ScoSoxR-down (5'-GCAGGTTTCCAGGGACAGGCAGCCGCAGCCGATGCAGTC-3') were used to create the upstream half, and the pair EcSoxR-up (5'-GCCTGTCCCTGGAAACCTGCCCCGTTGCGTAACCCGGGCGA-3') and EcSoxR-down (5'-GCGCCCTGGATCCGCTTTAG-3'; BamHI site underlined) were used to create the downstream half, which overlap by 18

complementary nucleotides. The second PCR from partially hybridized half templates were done with the primer pair ScoSoxR-up and EcSoxR-down to create the entire open reading frame for SWAP1 and SWAP2. The 462 bp PCR product was digested with NdeI and BamHI and cloned into pTac4 vector. The resulting plasmid vector transformed into *E. coli* soxR mutant. To prepare RNA, grown to exponential phase at OD₆₀₀ of 0.4~0.5. RNAs were purified by using acidic phenol after fixation with RNAprotect® Bacterial Reagent (QIAGEN) and performed S1 mapping analysis.

II.4.4. Electrophoretic mobility shift assay (EMSA) for SoxR-DNA binding.

DNA probes containing predicted SoxR binding sites were prepared by PCR using primer pairs as detailed below. For SCO2478, GM143F 5'- GGT GAC CGG TGC CTC CGA AC -3' and GM143R 5'- GGTGCGGTCGTCGTG TTCAC -3'; For SCO4266, GM127F 5'- CCTGACGGCGGTATCCCTCG -3' and GM127R 5'- CAGTCGGATGGCGTGCATGG-3'. Purified PCR products were labeled at 5' ends with [γ -P³²] ATP using T4 polynucleotide kinase. The binding reaction was carried out by incubating approximately 2.2 fmol of labeled DNA and varying amounts (0.5 ~5 pmol) of purified His-SoxR in 20 μ l reaction buffer [20 mM Tris-HCl (pH 7.8), 1 mM MgCl₂, 40 mM KCl, 2 mM DTT, 0.1 mg/ml BSA, 5% glycerol, and 0.1 μ g poly (dI-dC)] for 20 min at room temperature. The binding mixture was subjected to electrophoresis on a 5% native polyacrylamide gel at 100V in TBE running buffer (90 mM Tris-borate and 2 mM EDTA). The dried gels were exposed to imaging screens to quantify by phosphor image analyzer (FLA-2000, Fuji). For competition assay, either specific (unlabeled probe; 5- and 50-fold molar excess) or non-specific (pGEM3zf (+) plasmid digested with *HpaII*; 250- and 500-fold molar excess) were added to the binding reaction.

II.4.5. S1 nuclease mapping analysis.

To prepare RNA *S. coelicolor* cells were grown in liquid YEME media containing 10.3% sucrose and 5 mM MgCl₂ to OD₆₀₀ of 0.4 - 0.5. RNAs were purified by acidic phenol extraction, after fixation of cells with RNAprotect® bacterial reagent (Qiagen). To prepare RNA from *E. coli* and *P. aeruginosa*, cells were grown in LB to OD₆₀₀ of 0.4 - 0.5 before treatment with chemicals. Gene-specific S1 probes for *actII*-ORF4, *actA*, SCO2478, SCO7008, SCO1909, and SCO1178 were generated by PCR using *S. coelicolor* M145 genomic DNA as a template. The probes for *soxS* and PA2274 were generated by PCR using *E. coli* and *P. aeruginosa* genomic DNA as templates, respectively. The probe for *actII*-ORF4 spans from -92 (upstream) to +47 (downstream) nt position relative to the start codon, for *actA* from -114 to +69, for SCO2478 from -177 to +100, for SCO7008 from -162 to +138, for SCO1909 from -152 to +72, for SCO1178 from -168 to +73, for *soxS* -84 to +88 and for PA2274 -91 to +120. For each sample, RNA (50 - 100 µg) was hybridized at 50°C with gene-specific probes labeled with [γ -³²P]-ATP. Hybridization and S1 nuclease mapping were carried out according to standard procedures (Kieser *et al.*, 2000). Following S1 nuclease treatment, the protected DNA probes were loaded on 6% polyacrylamide gel containing 7 M urea. The signal was detected and quantified by BAS-2500 (Fuji).

II.5. Protein purification

II.5.1. Overproduction and purification of *S. coelicolor* SoxR protein from *E. coli*.

The entire coding region of the *soxR* gene was amplified from cosmid SC8F11 using mutagenic primers, SoxR-up (5'-GGTTCGAGCATATGCTCAGATTTC-3'; *Nde*I site underlined) and SoxR-down (5'-GACCGGGCCAGGATCCCGCG-3'; *Bam*HI site underlined). The 590 bp PCR product was

digested with *Nde*I and *Bam*HI and cloned into pET15b vector (Novagen). The resulting recombinant plasmid (pET15b::*soxR*) was transformed into *E. coli* BL21 (λ DE3) pLysS. To purify SoxR protein, transformant cells grown in LB at 37°C to OD₆₀₀ of 0.5 were induced with 1 mM (final concentration) isopropyl- β -D-thiogalactopyranoside (IPTG) for 3 hrs at 30°C. His-tagged SoxR protein was purified through nickel-charged NTA column (Novagen) as recommended by the manufacturer. Following dialysis to remove imidazole and excess nickel, the SoxR sample was concentrated by a centrifugal filter device (Millipore, 3,000 MW CO) and further purified through Superdex 75 column in FPLC system (ÄKTA standard, Amersham Biosciences). SoxR fractions were collected and further dialyzed against storage buffer (20 mM Tris-HCl, pH 7.8, 500 mM NaCl, 30% glycerol and 2 mM DTT). The concentration of purified SoxR protein was determined by Bradford method and was stored at -80°C.

II.5.2. Enzyme activity assay

II.5.2.1. β -Galactosidase (*LacZ*) assay

β -galactosidase activity was measured in whole cells with the addition of ONPG (o-nitrophenyl- β -D-galactopyranoside) after permeabilization of cells with SDS (sodium dodecyl sulfate)-chloroform (Miller, 1972). Cells were grown in LB medium up to an optical density of 0.2 ~ 0.5 at 600 nm and one of which was either untreated or treated with various concentration of RACs for 0.5 ~ 1 hour at 37°C. β -galactosidase activity was then assayed as described by Miller (1972) and was calculated in Miller units. Units of β -galactosidase activity were calculated by the following formula;

$$(1000 \times (A_{420} - 1.75 \times A_{550}) \times \text{dilution factor}) / (\text{time (min)} \times A_{600} (\text{culture}))$$

II.6. Biochemical assays

II.6.1. UV-visible absorption spectrometry

UV-visible absorption spectrums were collected using a UV-1650PC (Shimadzu) in the 250-800 nm wavelength range. Measurements were carried out at room temperature in Cuvette, stoppered 10 mm (Agilent Technologies).

II.6.2. Electron paramagnetic resonance (EPR) spectroscopy of SoxR

For whole-cell EPR measurements, *E. coli* XA90 (Ding & Demple, 1997) cells containing either pTac4 or pTac4-based recombinant plasmids that overproduce ScSoxR, EcSoxR, or PaSoxR under the control of IPTG-inducible tac promoter (Koo *et al.*, 2003) were grown in LB with chloramphenicol (34 µg/ml). When cells were grown to OD₆₀₀ of 0.20, 0.5 mM IPTG was added, and cultures were further incubated at 37°C for 2 h or more until OD₆₀₀ reached to 0.8 to 1.0. They were then left untreated or treated with redox-cycling drugs for 40 min. After treatments, cells were harvested, washed quickly with minimal salts (60 mM K₂HPO₄, 33.3mM KH₂PO₄, 7.6 mM (NH₄)₂SO₄, 2.3 mM Na₃C₆H₅O₇·7H₂O), and resuspended at 1/250th of the original culture volume in minimal salts containing 50% glycerol. Cell suspensions (300 µl each) were then transferred to EPR tubes and immediately frozen on dry ice. The expression level of SoxR in the soluble fraction of cells subjected to EPR analysis was confirmed on SDS-PAGE in a parallel experiment. EPR spectra of [2Fe-2S]⁺ clusters were obtained using a Varian E112 EPR spectrometer at the University of Illinois at Urbana-Champaign. The following settings were used throughout the measurement: microwave power, 1 mW; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 G at 100 KHz; time constant, 0.032; and sample temperature, 15 K.

II.6.3. Redox titration of SoxR

Purified SoxR protein was diluted to 10 μ M in TGDN500 buffer containing redox mediator safranin O (5 μ M) in a stoppered cuvette of 1-mm path length. The amount of oxidized SoxR was estimated by measuring absorption at 415 nm in a UV-1650PC spectrophotometer (Shimadzu). Redox titration was done by adding different amount of sodium dithionite at 25°C. The redox potential of the solution at each addition of sodium dithionite was measured with a combined platinum and Ag/AgCl electrode (HACH-MTC101-1) in an anaerobic chamber. The fraction of oxidized SoxR in each redox condition was calculated as described previously (Kobayashi & Tagawa, 2004).

II.7. Methods for bioinformatic analyses

II.7.1. Genome databases

All genetic information concerned to sequence and annotation of *S. coelicolor* was referred to ScoDB (<http://streptomyces.org.uk/>). Blast searches of genes in *S. coelicolor* were performed using *S. coelicolor* Blast Server in the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_coelicolor). Information of *E. coli* gene annotation was referred to Colibri Web Server (<http://genolist.pasteur.fr/Colibri/>) and SubtiList Web Server (<http://genolist.pasteur.fr/SubtiList/>) in the Institut Pasteur, respectively. Sequence data were available also in National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>).

II.7.2. Analysis of sequence and structure

Sequence alignment was performed by AlignX in Vector NTI Suite 8.0 (Invitrogen Co.). The structure of ScSoxR was predicted by SWISS-MODEL (Schwede *et al.*, 2003) with EcSoxR. Visualization of modeled structure and

comparative analysis with *E.coli* and *P. aeruginosa* SoxR were done using Vector NTI™ (Invitrogen Co.). Multiple sequence alignment of SoxR homologues was carried by Clustal W program in Vector NTI package. Representative SoxR homologues were selected from BLAST search, using EcSoxR, ScSoxR, PaSoxR as sequence queries. A phylogenetic tree was built by Vector NTI™ (Invitrogen) using the Neighbor Joining method (NJ). The distance from the nearest branch, point was indicated in parenthesis.

II.8. Site-specific mutagenesis of SoxR

To verify the various amino acid residues for superoxide sensing, template plasmid containing its own promoter and *soxR* wild type gene was constructed in the pGEM-Teasy. This construct was named as pSJ703. And then, selected target amino acids were exchanged into Ala or Ser by site directed mutagenesis, respectively. PCR was carried out with *PfuTurbo* DNA polymerase; denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 68°C for 7 min, 18 cycles. The amplification products were digested with *DpnI* at 37°C for 2 h to select for mutation-containing synthesized DNA and transformed to DH5 α . The sequences of the *soxR* variant genes were verified by automated DNA sequencing.

Table II-2. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant genotype and characteristics	Reference
<i>S. coelicolor</i>		
M145	SCP1-SCP2-	Kieser <i>et al.</i> , 2000
M145 Δ SoxR	M145 with a deletion in soxR	Shin <i>et al.</i> 2011
M145 Δ SoxR::ScSoxR	M145 Δ SoxR::pSET162-pScSoxR-ScSoxR	This study
M145 Δ SoxR::EcSoxR	M145 Δ SoxR::pSET162-pScSoxR-EcSoxR	This study
M145 Δ SoxR::PaSoxR	M145 Δ SoxR::pSET162-pScSoxR-PaSoxR	This study
<i>E. coli</i>		
XA90	K-12 Δ (lac-pro) XII ara nalA argE(Am) thi Rif (F' lacI ^{q'} ZY proAB)	(Hidalgo & Demple, 1994)
BL21 (λ DE3) pLysS	<i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHI ΔEcoRI-B int::(lacI:: PlacUV5::T7 gene1) i21 Δnin5</i>	Lab culture stock
GC4468	(<i>argF-lac</i>) 169 <i>rpsL sup</i> (Am)	Lab culture stock
GC4468 Δ soxR	(<i>argF-lac</i>) 169 <i>rpsL sup</i> (Am) \square soxR::Kan ^r	This study
MS1343	GC4468, <i>soxSp::lacZ</i> , Amp ^r	Koo <i>et al.</i> 2003.
MS1343 Δ soxR	GC4468, <i>soxSp::lacZ</i> , Amp ^r Δ soxR::Kan ^r	Koo <i>et al.</i> 2003.
ET12567	F' <i>dam13::Tn9 dcm6 hsdM hsdR recF143::Tn10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL hisG4 tsx-78 mtl-1 glnV44</i>	(Macneil <i>et al.</i> , 1992)
<i>P. aeruginosa</i>		
PA14	WT, Non-infectious strain	D. Newman
Plasmids		
pSET162	insertion of a thiostrepton resistance marker at SphI site of pSET152 (Apramycin ^r <i>lacZa</i> MCS reppUC)	(Kim <i>et al.</i> , 2006)

pSET162-pScSoxR-ScSoxR	pSET162 contain promoter of ScSoxR with <i>Sc soxR</i> gene	This study
pSET162-pScSoxR-EcSoxR	pSET162 contain promoter of ScSoxR with <i>Ec soxR</i> gene	This study
pSET162-pScSoxR-PaSoxR	pSET162 contain promoter of ScSoxR with <i>Pa soxR</i> gene	This study
pTac4	pTac1 Amp ^r replaced with Chl ^r	This study
pTac4-ScSoxR	pTac4 vector contain <i>Sc soxR</i> gene	This study
pTac4-ScSoxR-truncated	pTac4 vector contain <i>Sc soxR</i> C-terminal truncated gene	This study
pTac4-EcSoxR	pTac4 vector contain <i>Ec soxR</i> gene	This study
pTac4-PaSoxR	pTac4 vector contain <i>Pa soxR</i> gene	This study
pTac4-MsSoxR	pTac4 vector contain <i>M. smegmatis soxR</i> gene	This study
pET15b	N-terminally histidine-tagged	Novagen
pET15b-ScSoxR	N-terminally 6 histidine-tagged <i>S. coelicolor soxR</i> gene in pET15b	This study
pET15b-EcSoxR	N-terminally 6 histidine-tagged <i>E. coli soxR</i> gene in pET15b	This study

CHAPTER III

RESULTS

III.1. Characterization of *S.coelicolor* [2Fe-2S] SoxR protein

III.1.1. In vitro properties of SoxR wild type and cysteine to serine substitution mutant proteins

In order to characterize the SoxR wild type and SoxR cysteine to serine mutant protein, the coding region of the SoxR gene was amplified from the *S. coelicolor* genomic DNA. Amplified products were cloned into pET15b (Novagen). Total molecular weight of Wild-type SoxR (pET15b-ScSoxR) or the respective mutant proteins encoded by pET15b-ScSoxR-Cys-Ser is about 21 kDa. Overexpression was carried out in *E. coli* BL21 (DE3) with 1 mM (final concentration) isopropyl- β -D-thiogalactopyranoside (IPTG) for 6 hrs at 30°C. Overexpressed SoxR wild type and SoxR cysteine to serine substitution mutant proteins were purified by using Ni-NTA column. SoxR wild type and SoxR cysteine to serine mutant proteins were eluted by elution buffer containing 300 mM imidazole and visualized on SDS-PAGE by comassie-blue staining (Fig. III-1).

III.1.2. Dimerization of SoxR

In order to determine the oligomeric status of SoxR wild type and SoxR cysteine to serine substitution mutant proteins in solution the following steps were monitored. Initial investigations using SDS-PAGE analyses and subsequent size-exclusion chromatography experiments indicated the existence of a band corresponding to the dimer (Fig. III-2a). To investigate the ability of SoxR to form dimers and multimers, a SDS-PAGE with hot and cold sample mixer experiment was performed. The results showed efficient cross-linking and the formation of multimers, out of which the dimeric form was predominant (Fig. III-2a). Leading to a consideration that SoxR interaction is highly cooperative. The process of multimerization was also examined using

size-exclusion chromatography experiments. Fig. III-2b shows size-exclusion chromatography results similar with SDS-PAGE experiments.

III.1.3. UV-VIS absorption spectrum and EPR characteristic of [2Fe-2S]-containing proteins

The UV-VIS absorption spectrum of air-oxidized ScSoxR indicated a peak at 420 nm and two shoulders at 450 and 550 nm, strongly suggesting a cluster characteristic of [2Fe-2S] where as peaks which disappeared upon the addition of sodium dithionite (Fig. III-3). To assess the redox state of SoxR, we performed EPR spectroscopic analysis using whole cells. EPR spectroscopy has been used to analyze the redox state of the overproduced proteins containing an iron-sulfur cluster in intact cells, since it can determine the unpaired electron of the Fe-S cluster (Johnson *et al.*, 1985), and has been applied successfully to monitor the redox state of SoxR *in vivo* (Ding & Demple, 1997; Gaudu *et al.*, 1997). The [2Fe-2S] cluster of SoxR produces a characteristic EPR spectrum in its reduced form ($[2\text{Fe-2S}]^+$, which disappears on oxidation to $[2\text{Fe-2S}]^{2+}$ (Hidalgo *et al.*, 1995). Since only a small amount of SoxR exists in wild-type cells (<100 molecules per cell), spectroscopic observation *in vivo* requires the overproduction of SoxR. We overproduced SoxR protein and SoxR cysteine to serine mutant in the wild type, and confirmed that similar amounts of SoxR were present in the soluble fraction, as judged by SDS-PAGE. The X-band EPR spectra from these cells were recorded at 96K as described in materials and methods. SoxR in wild-type cells demonstrated its characteristic spectrum as a reduced form (Fig. III-4). The intensity of the EPR signal completely disappeared in SoxR cysteine to serine mutant and vector control. Since only the reduced form of the [2Fe-2S] cluster produced the EPR signal, the data clearly demonstrated that the *S. coelicolor* SoxR have [2Fe-2S] cluster and these might be involved in gene regulation.

III.1.4. The [2Fe-2S] clusters in SoxR are essential for transcriptional activity

[2Fe-2S] clusters are essential for *E. coli* SoxR activity (Bradley *et al.*, 1997) and, although this has not been formally demonstrated (by mutagenic analysis), presumably also for the activity of the pseudomonad SoxR proteins. In these organisms, redox-active agents oxidize SoxR's [2Fe-2S] clusters to activate the protein. To confirm if SoxR is similarly redox regulated in *S. coelicolor*, we created a [2Fe-2S]-deficient mutant by replacing the cysteine to serine. The equivalent mutation in *E. coli* SoxR results in elimination of [2Fe-2S] clusters and an inability to activate *soxS* transcription (Bradley *et al.*, 1997). We confirmed the absence of [2Fe-2S] clusters by monitoring the EPR spectrum of the purified cysteine to serine protein. While wild-type SoxR produced an EPR spectrum characteristic of [2Fe-2S]-containing proteins, the cysteine to serine mutant protein did not produced one (Fig. III-4). Gel shift assays (EMSA) conducted with purified histidine-tagged proteins showed that while the cysteine to serine mutant protein retains the ability to bind to the promoters of SCO2478 and SCO4266 but binding efficiency is lower than wild type. It binds with a lower level of affinity than wild-type SoxR (Fig. III-5). This has also been observed with cluster-deficient *E. coli* SoxR, which exhibits a promoter-binding defect both in vivo and in vitro (Chander & Demple, 2004; Chander *et al.*, 2003). We introduced the cysteine to serine substitution *soxR* mutant gene into the *soxR* strain via the pSET162 vector to allow chromosomal integration of the gene via the att site and monitored the transcription on treatment of actinorhodin of *soxR* itself, SoxR target genes, SCO1909 and *actR* as a positive control, by S1 mapping analysis. The results indicated in Fig. III-6 show that while complementation with wild-type *soxR* restored upregulation of SCO1909, the cysteine to serine substitution mutant was unable to rescue the Δ *soxR* defect. Therefore, [2Fe-2S] clusters are critical cofactors required for transcription.

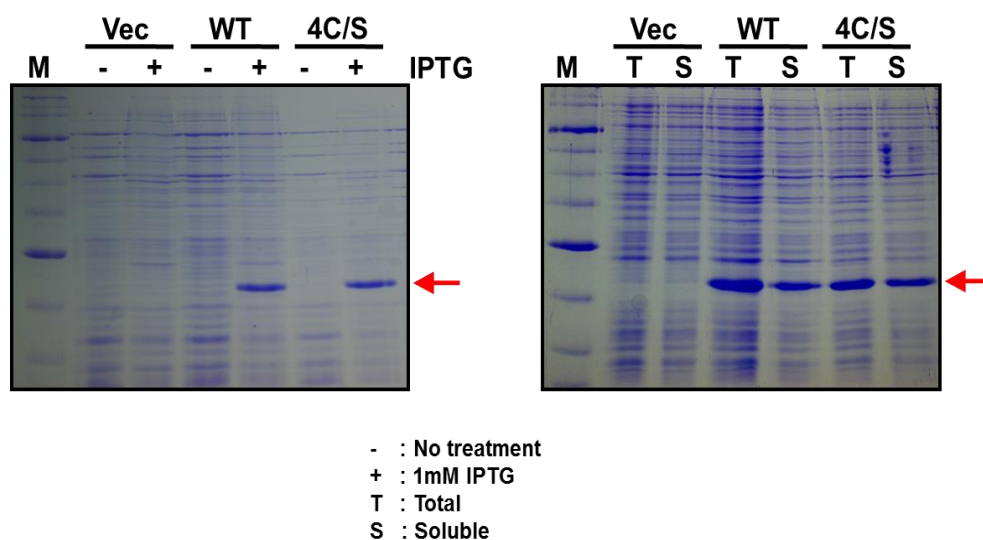


Fig. III-1. Overproduction profiles of SoxR and cysteine to serine substitution mutant proteins by IPTG treatment in *E. coli*. *E. coli* BL21 (DE3) pLysS cells harboring pET15b 6 his-tag were grown in 3 ml LB to OD₆₀₀ of 0.5 and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. After harvest, cells were resuspended in 30 μl 1X SDS loading buffer. Resuspended samples were analyzed on 13% SDS-PAGE and induced protein bands as well as solubility are visualized by coomassie staining. Black arrows and M indicate bands of the induced SoxR and cysteine to serine substitution proteins by IPTG and standard size marker, respectively. Total molecular weight of SoxR is about 21 kDa.

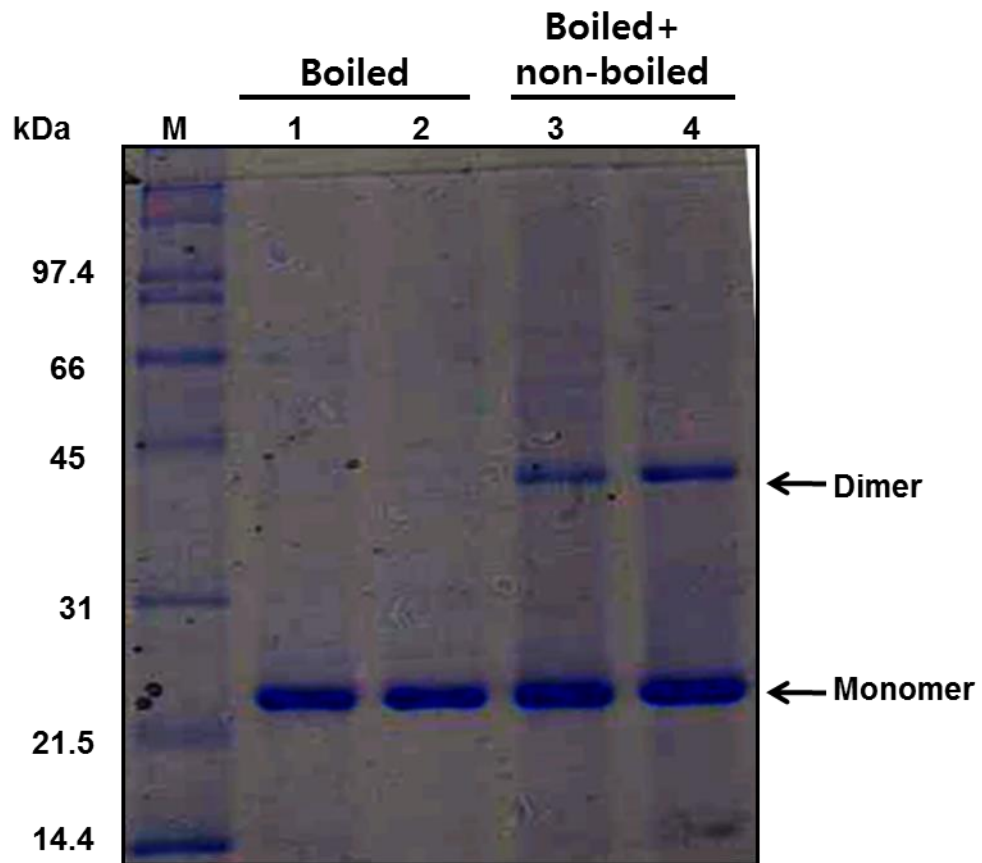


Fig.III-2.a. Oligomeric states of SoxR in SDS-PAGE. SDS Gel electrophoresis of purified SoxR proteins. Wild-type and cysteine to serine substitution mutated SoxR proteins (5 μ g) were subjected to 13% SDS/PAGE. To visualize dimeric forms better, boiled samples in SDS-loading dye were mixed with non-boiled protein before loading. Lane M, molecular mass markers; lanes 1 and 3 is SoxR; lane 2 and 4 is SoxR cysteine to serine mutant Protein.

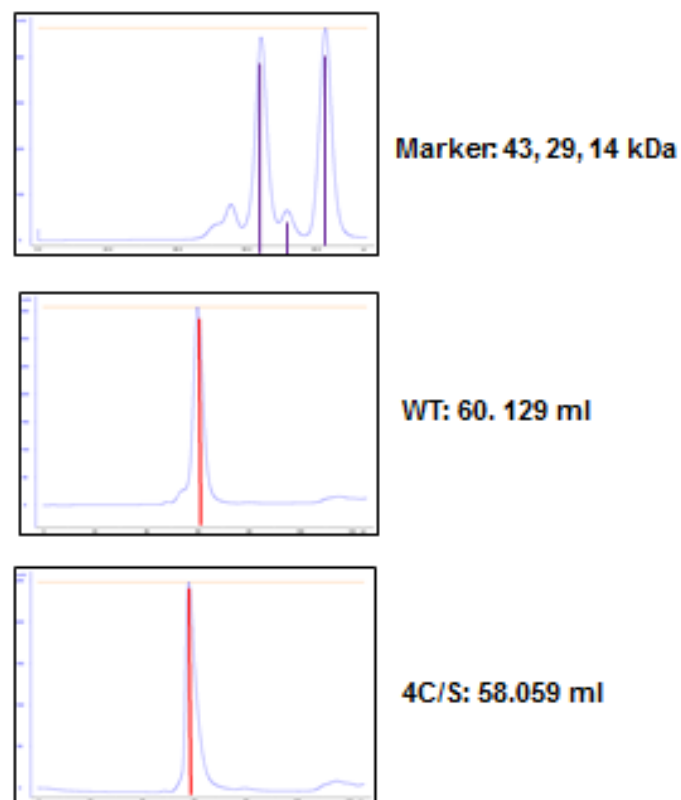


Fig. III-2.b Determination of oligomeric state of SoxR by size-exclusion chromatography. Wild-type and cysteine to serine substitution mutant proteins were purified by size-exclusion chromatography on the basis of molecular wt. standard marker are used 43, 29 and 14 kDa. Wild-type are collected at 60.19 ml and 4C/S mutated SoxR at 58.05 approximately same with wild type, showing that dimer nature of proteins.

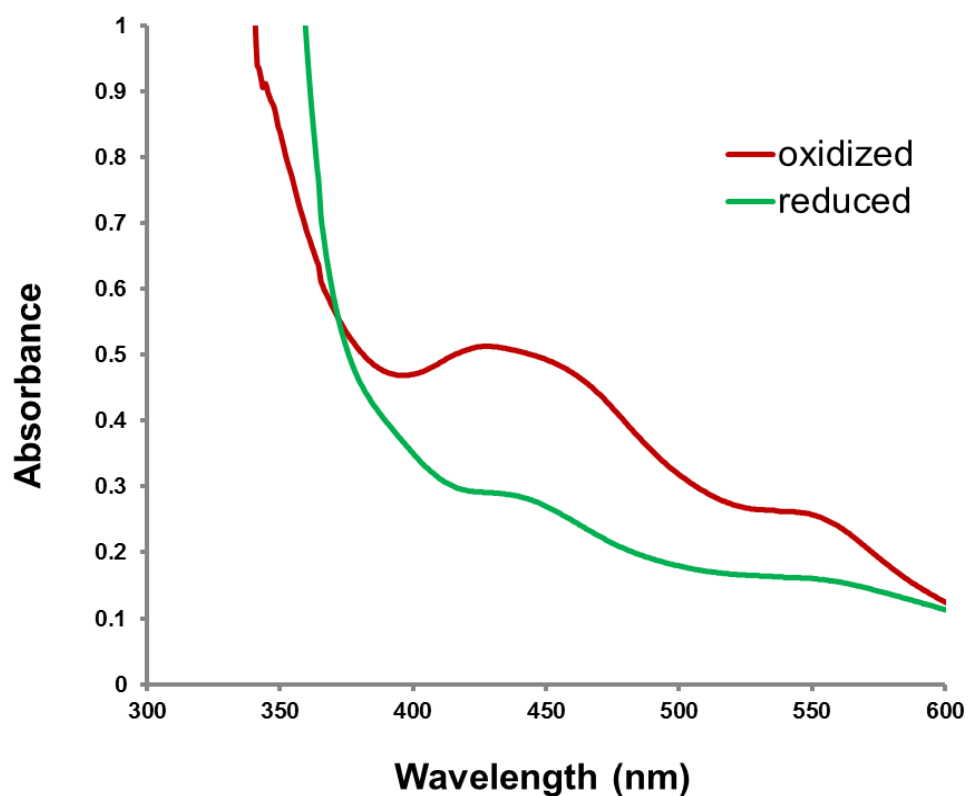


Fig. III-3. Absorption spectrum of *S.coelicolor* SoxR protein. *S.coelicolor* SoxR protein purified from *E. coli* were resuspended to 20 μ M each in 20 mM Tris-HCl (pH 7.8) containing 500 mM KCl, 10% glycerol, and 1 mM DTT in stoppered cuvettes and absorption spectra of oxidized and reduced ScSoxR were measured with UV-visible spectrophotometer.

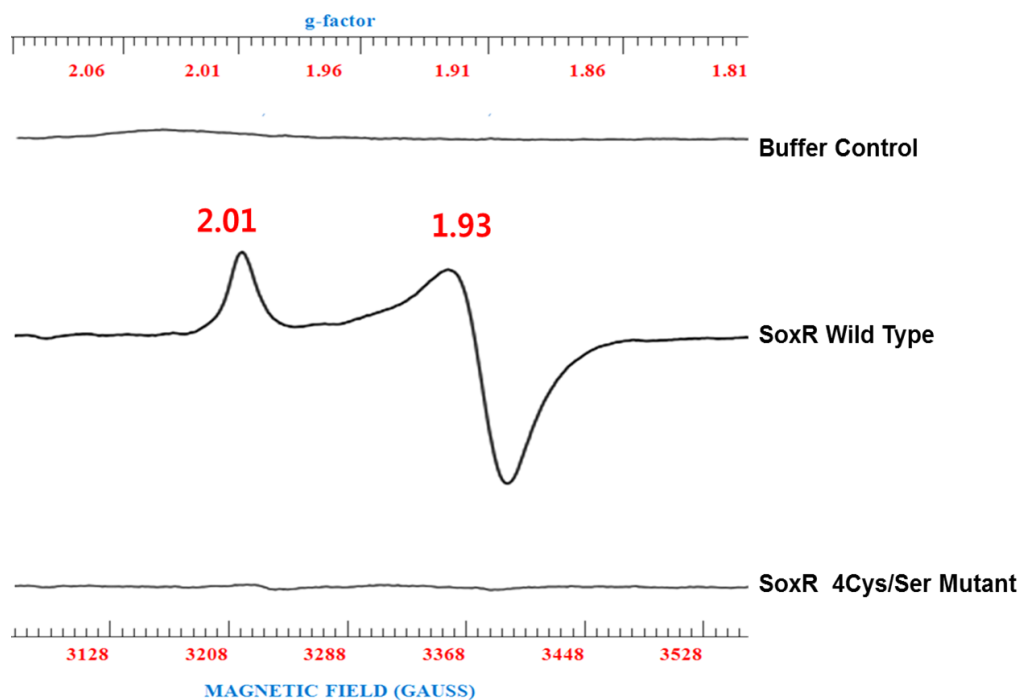


Fig. III-4. Whole cell EPR analysis of overexpressed SoxRs in *E. coli* BL21. The redox status of the [2Fe-2S] clusters in *S.coelicolor* SoxR Wild-type and cysteine to serine substitution mutated proteins, which were overproduced in *E. coli*, were measured by EPR. The pET15b-histagged-based recombinant plasmids used and were introduced into *E. coli* BL21 wildtype cells. Each transformant strain was grown aerobically in LB medium to an OD₆₀₀ of 0.5, when IPTG was added, followed by further incubation at 37°C for more than 4 h. After washing and resuspension, intact cells were transferred to EPR. EPR measurements were performed at 96 K .

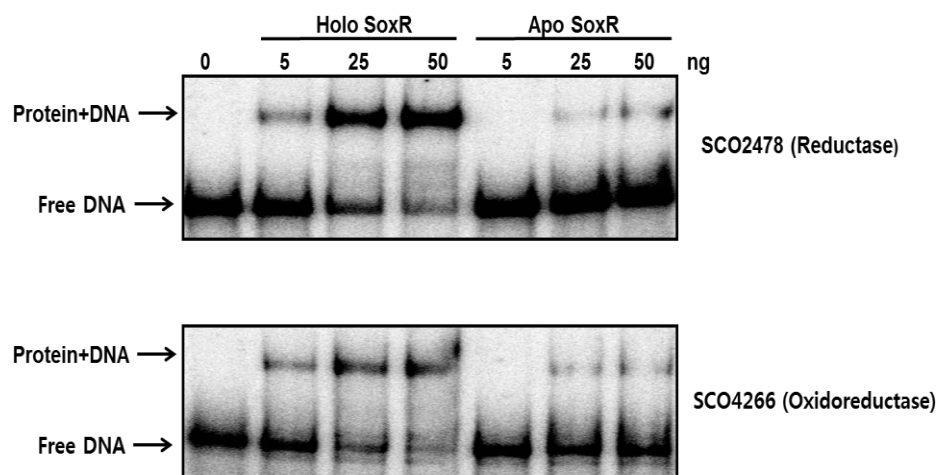


Fig. III-5. DNA binding assays with SCO2478 and SCO4266 promoters and purified SoxR protein. Bandshift assay using a 143bp (SCO2478) and 127bp (SCO4266) 10 ng per reaction carrying the SCO2478 and SCO4266 promoter. apo-SoxR or holo [2Fe-2S] SoxR as indicated in 20 mM Tris-Hcl pH 7.0, 500 mM NaCl buffer. The apo-form of the protein is unable to bind to the SCO2478 and SCO4266 promoter indicating that the cluster is required for DNA binding activity.

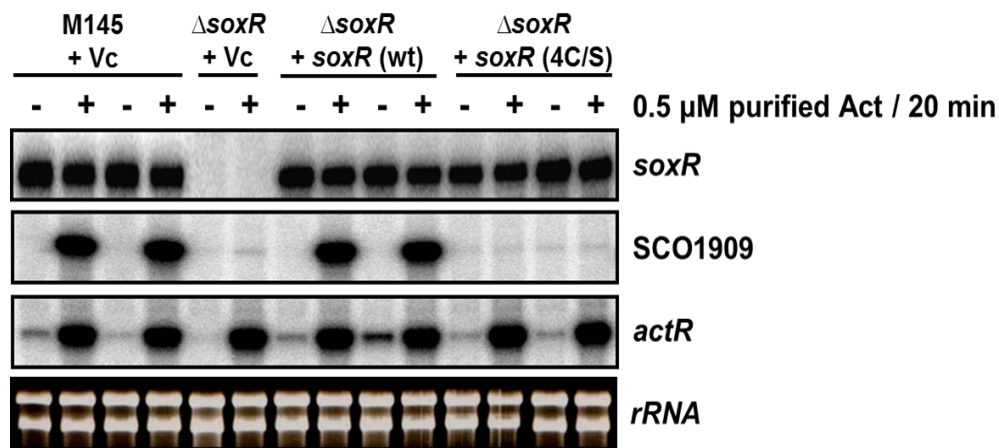


Fig. III-6. Contribution of Fe-S cluster to actinorhodin dependent regulation of SoxR. The 4C/S SoxR mutant does not complement the M145 Δ soxR strain. Activation profile in *S. coelicolor* cell background, genes for ScSoxR and 4C/S SoxR, were cloned in the pSET-152-derived integration vector pSET162 and introduced into the Δ soxR mutant strain of *S. coelicolor*. Since all these SoxRs share similar recognition sequences, we monitored the amount of SoxR target gene transcripts in *S. coelicolor*, as indicators of SoxR activation. Actinorhodin (Act; 500 nM), was added to exponentially growing *S. coelicolor* cells containing pSET162 vector, pSET162-ScSoxR, pSET162-4C/S SoxR integrated at the att site in the chromosome. Gene-specific probes for SoxR target SCO2478 were used for S1 mapping.

III.2. Comparative study of SoxR activation by redox-active compounds

III.2.1. Induction of ScSoxR by both natural and xenobiotic redox active compounds in *S. coelicolor*

As a first step toward understanding the role and activation behavior of SoxR in *S. coelicolor*, we examined the effect of various redox-active compounds (RACs): three natural metabolites (actinorhodin, pyocyanin, toxoflavin) and five xenobiotic redox-cycling agents (phenazine metho-sulfate, paraquat, plumbagin, menadione, and menadione sodium-bisulfite) (Fig. III-7). The effective concentration ranges for ScSoxR activation were determined (Fig. III-8.a). exponentially growing cells were treated with RACs of varying concentrations for 30 min before RNA was isolated. S1 mapping was performed to quantify transcripts from a SoxR target gene (SCO2478), encoding a putative NADPH-dependent flavin reductase. The results demonstrated that as little as 20 nM γ -actinorhodin induced SoxR target gene expression, with maximal induction occurring between 200 nM and 500 nM (Fig. III-8.a). Pyocyanin, a toxic blue phenazine pigment produced from *P. aeruginosa*, activated ScSoxR in low-micromolar doses and did so maximally at 25 to 100 μ M. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a yellow pigment originally isolated from plants of genus *Plumbago*, activated SoxR in a narrow range of concentrations, with maximal induction at 25 to 50 μ M. Menadione (2-methyl-1,4-naphthoquinone), a chemically synthesized naphthoquinone derivative, also activated SoxR in a narrow range of concentrations, with maximal induction at 200-300 μ M. The water-soluble salt form of menadione (menadione sodium bisulfite; MDs) was not able to activate SoxR at any concentration examined, ranging from 5 μ M to 1 mM (Fig. III-8.b). This was not due to a cell-permeability barrier, as demonstrated

below (Fig. III-11). Phenazine methosulfate (PMS), a chemical phenazine derivative, activated SoxR in a broad concentration range from 0.1 μ M to 1 mM, maximally at 0.3 to 50 μ M. We examined the effect of methyl viologen (paraquat; PQ) in the concentration range of 5 μ M to 1 mM, and found that it did not activate SoxR (Fig. III-8.b). Again, this insensitivity was not due to permeability barrier as shown below (Fig. III-11). Longer treatment of RACs inhibited growth by more than two-fold within 2 h at higher concentrations (> 300 nM actinorhodin, > 50 μ M plumbagin, 300 μ M menadione, >100 μ M PMS; Fig. III-8.c).

Why treatment with high concentrations of PL, MD, and PMS for 30 min were not effective to induce SoxR target genes is not clear. We found that these treatments do not always inhibit mRNA synthesis, since some inducible promoters are activated by the treatment (Fig. III-8.d). It is conceivable that high dose of these oxidants limit the supply and/or function of transcriptional machinery for the SoxR-dependent promoters, even when SoxR is activated. Currently, it is not known which sigma factor(σ) directs transcription from SoxR-activated promoters, out of more than 60 sigma factors predicted in *S. coelicolor*. It is also conceivable that SoxR no longer is maintained as an active oxidized form at high concentrations of RACs, which could generate metabolic byproducts such as ROS that could facilitate breakdown of the [Fe-S] cluster.

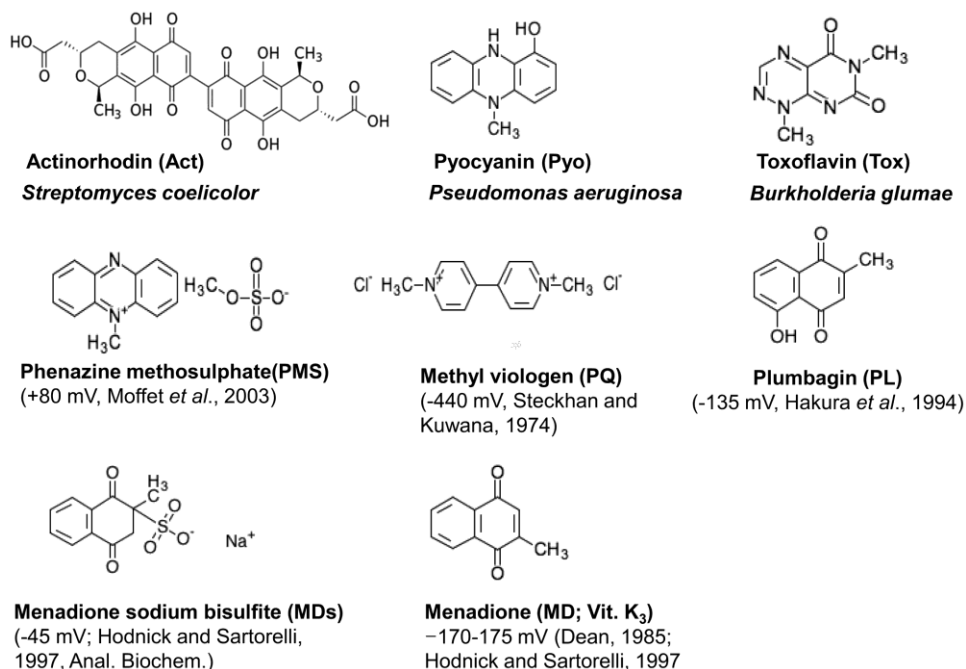
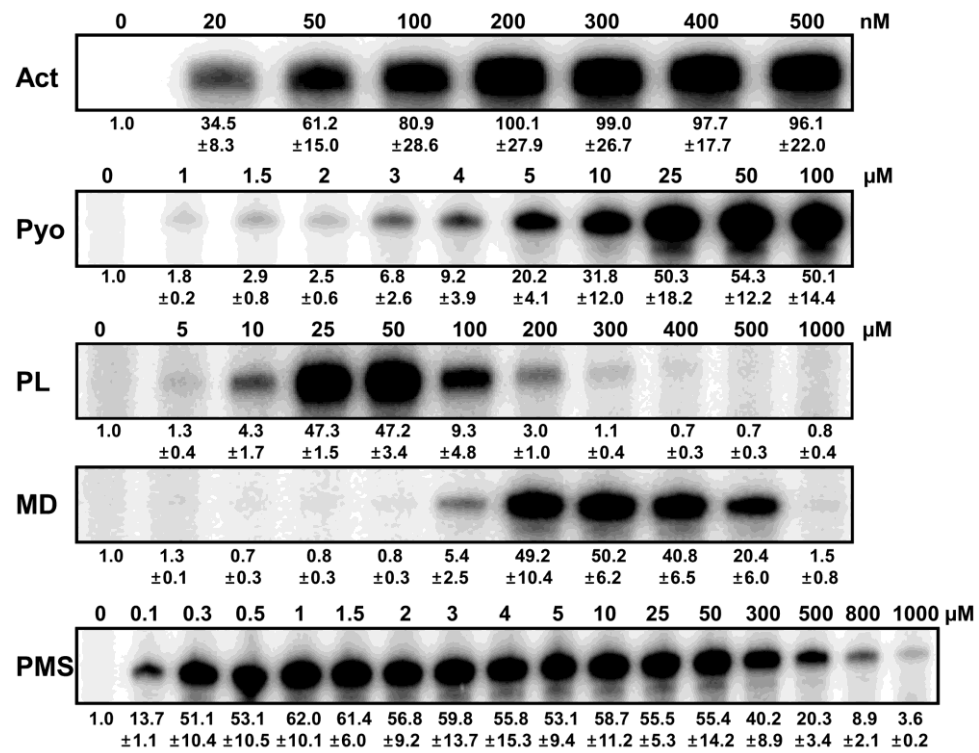
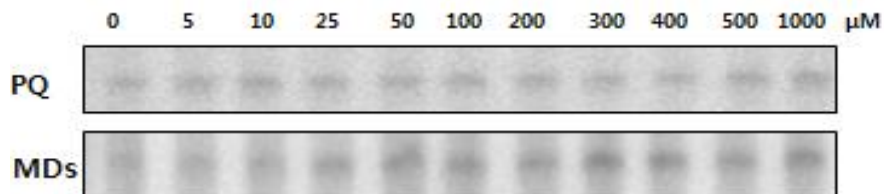


Fig. III-7. Chemical structures of redox-active compounds (RACs) examined in this study. Three natural metabolites from *S. coelicolor* (actinorhodin), *P. aeruginosa* (pyocyanin), and *Burkholderia glumae* (toxoflavin), and five xenobiotic redox-cycling agents were examined. The reported reduction potentials of the xenobiotics are indicated in parentheses. The reduction potential for paraquat (PQ, methyl viologen) is indicated for the pair PQ^{2+}/PQ^{1+} , since reduction of PQ^{1+} to PQ^0 has a much lower potential and thus is irrelevant (Steckhan and Kuwana, 1974).

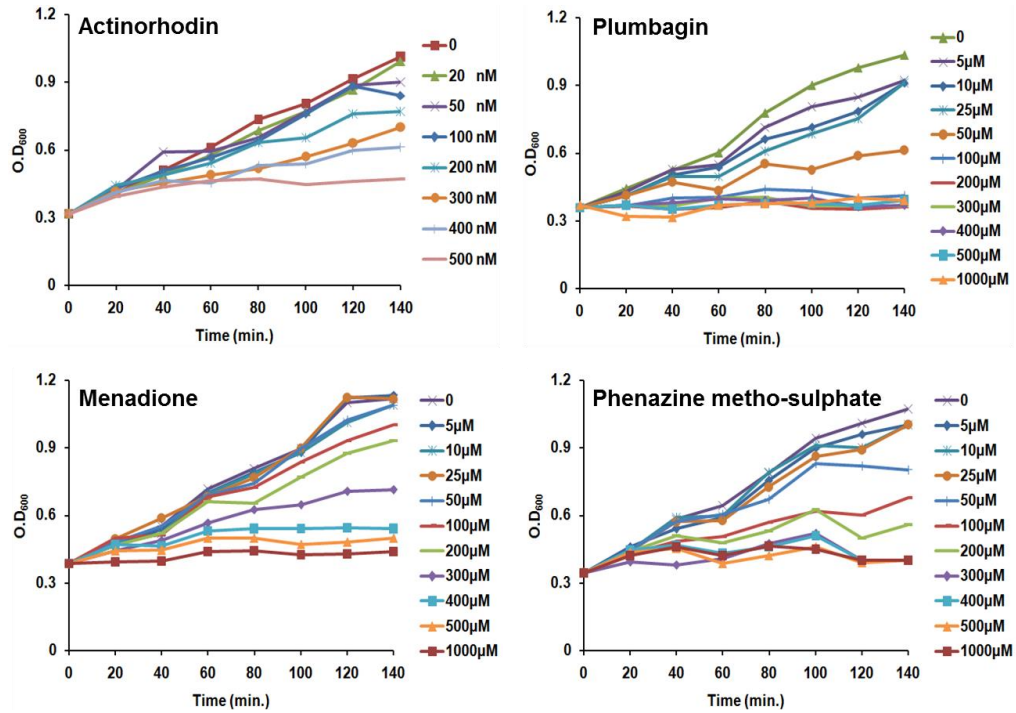
A



B



C



D

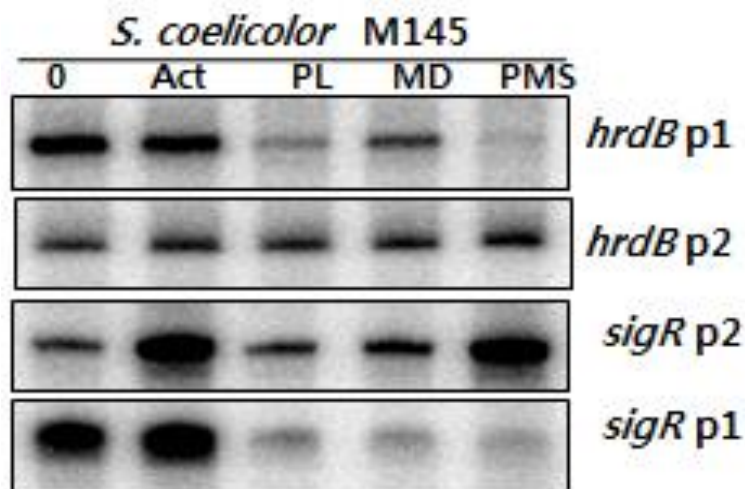


Fig. III-8. The effective concentration range of RACs to activate SoxR in *S. coelicolor*. (A) Varying concentrations of RACs [0 - 0.5 μ M of actinorhodin (Act), 0 - 0.1 mM of pyocyanin (Pyo), 0 - 1 mM of plumbagin (PL), menadione (MD; same amounts as PL), and phenazine methosulfate (PMS)] were added to exponentially grown *S. coelicolor* wild type cells (OD \sim 0.4 in YEME) for 30 min. (B) 0 - 1 mM of methy viologen (PQ) and menadione bisulfite (MDs) . To assess SoxR activation, the amount of its direct target gene transcript (SCO2478) was analyzed by S1 nuclease mapping. The level of gene expression relative to the untreated level was quantified from at least three independent experiments and is presented at the bottom of each data set. (C) Growth pattern of M145 observed after treatment with various redox compounds (D) Activation profile in *S. coelicolor* cell background. Genes for house keeping genes such as *hrdB* and *sigR*, we monitored the amount of house keeping gene transcripts in *S. coelicolor*, as indicators of *hrdB* and *sigR* activation at higher concentrations (> 300 nM actinorhodin, > 50 μ M plumbagin, 300 μ M menadione, >100 μ M PMS for 30 min were used for S1 mapping.

III.2.2. SoxR protects cells from the growth-inhibiting effects of SoxR-inducing chemicals

Whether the activation of SoxR plays any protective function against toxic inducing chemicals was examined by monitoring cellular growth in liquid media (YEME) through optical-density measurements. For this purpose, exponentially growing *S. coelicolor* wild type (M145) and $\Delta soxR$ mutant cells (at OD₆₀₀ ~0.3) were treated with lower doses of actinorhodin (100 nM) or plumbagin (25 μ M) for 30 min, and they were then either unchallenged or challenged with higher concentrations of the same compound. The results in Fig. III-9. Clearly demonstrate that the $\Delta soxR$ mutant experienced more severe growth inhibition than the wild type by these compounds. Thus the activation of SoxR by RACs in *S. coelicolor* confers resistance toward these chemicals.

III.2.3. Differential sensitivity profile of SoxRs toward RACs in *S. coelicolor*, *E. coli*, and *P. aeruginosa*

We then examined the induction of SoxR regulon by a variety of RACs presented in Fig. III-7. exponentially grown wild type cells of *S. coelicolor* (M145), *E. coli* (GC4468), and *P. aeruginosa* (PA14), at OD₆₀₀ ~0.4–0.5 in YEME or LB liquid medium, were treated for 30 min with actinorhodin (Act; 200 nM), pyocyanin (Pyo; 10 μ M), toxoflavin (Tox; 20 μ M), phenazine methosulfate (PMS; 50 μ M), paraquat (PQ; 200 μ M), plumbagin (PL; 25 μ M), menadione sodium-bisulfite (MDs; 500 μ M) or menadione (MD; 350 μ M) before cell harvest. The activation of SoxR was estimated by the quantification of transcripts from a native target gene in each organism by S1 mapping. Results in Fig. III-10 demonstrated that each organism responds to RACs in distinctly different ways. *E. coli* and *P. aeruginosa* did not respond to γ -actinorhodin by activating SoxR. This insensitivity, however, was due to a permeability barrier that prevented γ -actinorhodin from entering these

organisms, as described below (Fig. III-11). The SoxR system in *E. coli* and *P. aeruginosa* responded to all the other compounds that were examined, albeit with varying degree of induction. Even though PQ and MDs did not activate SoxR in *S. coelicolor*, they were effective in activating SoxRs in *E. coli* and *P. aeruginosa*. Since the SoxR induction, profile in each organism is the combined result of permeability and *in vivo* effectiveness of each RAC, a uniform cellular environment is necessary to examine the species-specific activation behavior of each SoxR.

III.2.4. Activation profile of three SoxR species expressed in *S. coelicolor* or in *E. coli* by various RACs

We then constructed recombinant strains of *S. coelicolor*, each of which expresses ScSoxR, EcSoxR, or PaSoxR from a chromosomally integrated gene in the $\Delta soxR$ background. Either the wild type strain or a $\Delta soxR$ mutant with an integrated parental vector (pSET162) was examined in parallel. Cells in mid-exponential culture (at OD₆₀₀ of ~ 0.4-0.5) in YEME liquid media were treated with RACs for 30 min, and expression of four SoxR target genes encoding a putative NADPH-dependent reductase (SCO2478), an ABC transporter (SCO7008), a monooxygenase (SCO1909), and a hypothetical protein (SCO1178) was then examined by S1 mapping. As a control for actinorhodin-specific gene induction, we examined RNAs from the actinorhodin gene cluster, *actA* encoding actinorhodin transporter and *actII-ORF4* encoding pathway-specific gene activator. The results in Fig. III-11.a. demonstrated that Act (500 nM) and Pyo (10 μ M) were effective in activating ScSoxR, whereas PQ (200 μ M) was not. All three compounds were effective in activating EcSoxR as well as PaSoxR, as judged by induction of all four-target genes. These experiments clearly demonstrate that the inability of γ -actinorhodin to activate SoxR in native *E. coli* and *P. aeruginosa* cells is most

likely due to permeability problems. Effective activation of PaSoxR by paraquat in *S. coelicolor* coincides with what was observed in its native host.

We then exploited a *LacZ* reporter system in *E. coli* to monitor activation behavior of each SoxR species in another identical cellular background. Each *soxR* gene was cloned in the pTac4 vector and was introduced into an *E. coli* *soxR* mutant that harbors a *soxS* promoter::*lacZ* fusion gene. Transformed cells were grown to exponential phase and treated with either 50 μ M PMS or 200 μ M PQ for 1 h. *LacZ* activity was then measured. Fig. III-11.b shows that EcSoxR and PaSoxR were effectively induced by both PQ and PMS, whereas ScSoxR was induced only by PMS. The absolute value of *LacZ* activity was relatively low in ScSoxR-containing *E. coli* strain; however, the degree of induction was about 18-fold, as high as for EcSoxR and PaSoxR. Thus the activation profiles of SoxR species that had been observed in the *S. coelicolor* cellular environment were reproduced in the *E. coli* background.

We examined a broader range of RACs in the *S. coelicolor* cellular environment as described in Fig. III-11.a. We found that toxoflavin activated all three SoxR species (Fig. III-12) and that menadione sodium sulfite (MDs) activated EcSoxR and PaSoxR while being ineffective for ScSoxR (Fig. III-13). Examination of NO-generating compounds (SNP, DETA, and GSNO) demonstrated that ScSoxR was not activated by any of them, whereas EcSoxR was activated by all of them and PaSoxR was activated efficiently by SNP but not as well by other compounds (Fig. III-14). All these results demonstrate that the three SoxR species show species-specific profiles of responses toward RACs. Overall, EcSoxR and PaSoxR both respond to a broader spectrum of oxidants, including paraquat, than does ScSoxR, which does not respond to PQ (a weak oxidant of low redox potential), MDs (a salt form of quinone with relatively high redox potential), or nitrosylating agents.

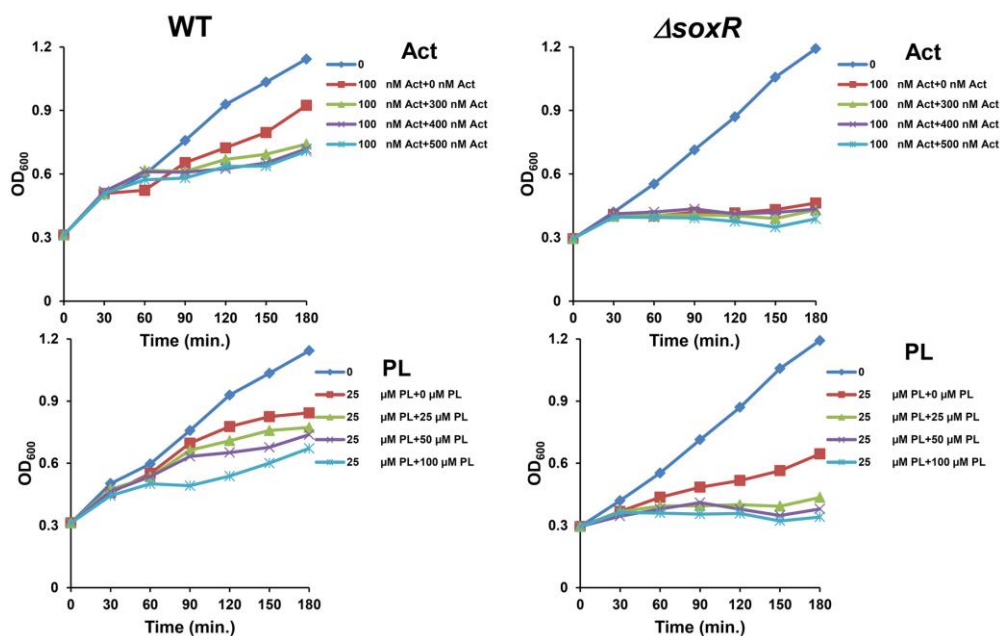


Fig. III-9. Role of SoxR in protecting *S. coelicolor* cells against actinorhodin and plumbagin. YEME liquid medium was inoculated with 10^8 spores of the *S. coelicolor* M145 wild type and Δ soxR mutant strains and was shaken at 180 rpm in an incubator at 30°C. When cultures reached mid exponential phase ($OD_{600} \sim 0.3 - 0.4$), either actinorhodin (Act; 100 nM) or plumbagin (PL; 25 μ M) was added. After 30 min of inducing treatment, higher amounts of the same compounds (0, 300, 400, 500 nM Act, or 0, 25, 50, 100 μ M PL) were added to the culture. Cell growth was subsequently monitored by measuring OD at 600 nm. Growth of non-treated cells was monitored in parallel. The data sets that are shown are representative of four independent experiments for each compound.

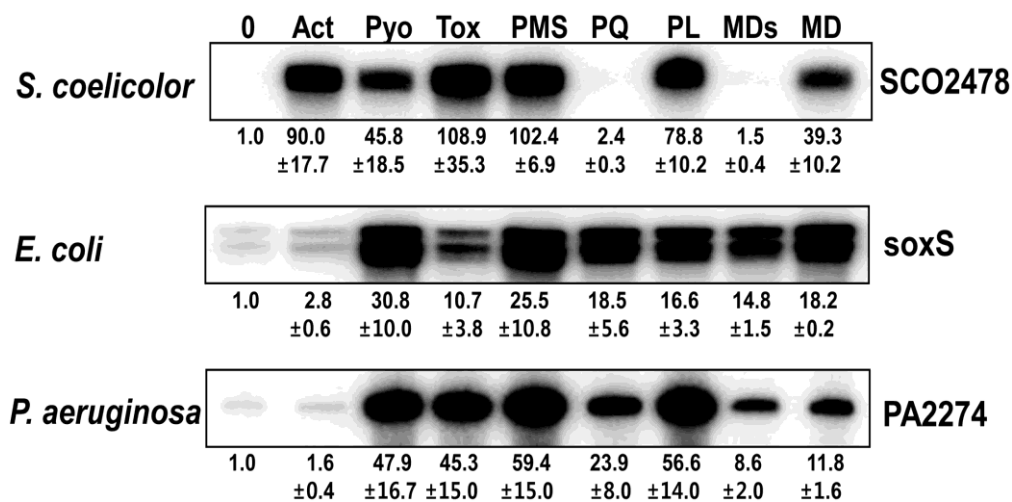


Fig. III-10. Reactivity of SoxRs with a variety of RACs in wild type *S. coelicolor* (M145), *E. coli* (GC4468), and *P. aeruginosa* (PA14) cells. Exponentially grown wild type cells ($OD_{600} \sim 0.4$ to 0.5) were treated with RACs for 30 min: Act 200 nM, Pyo 10 μ M, Tox 20 μ M, PMS 50 μ M, PQ 200 μ M, PL 25 μ M, MDs 500 μ M and MD 350 μ M. The amount of SoxR target transcripts was then analyzed by S1 mapping for *S. coelicolor* (SCO2478), *E. coli* (soxS), and *P. aeruginosa* (PA2274). The soxS mRNA from *E. coli* produces two protected bands, the smaller of which is most likely generated from a processed species as observed by (Wu and Weiss, 1991). Relative expression levels were obtained from at least three independent experiments and are presented at the bottom of each data set.

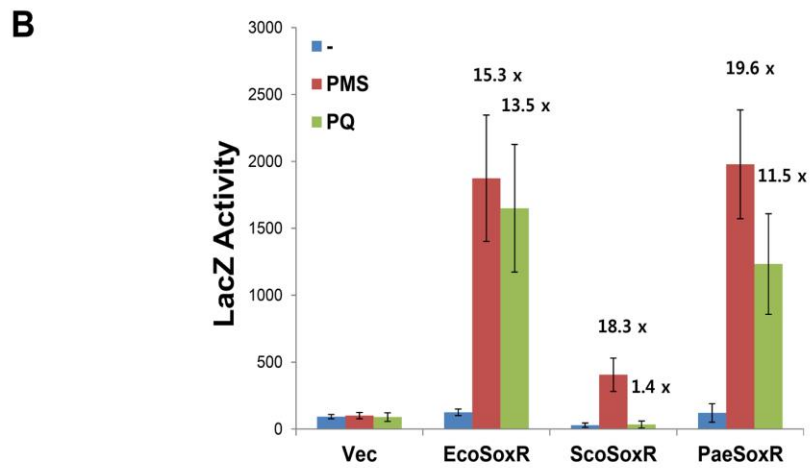
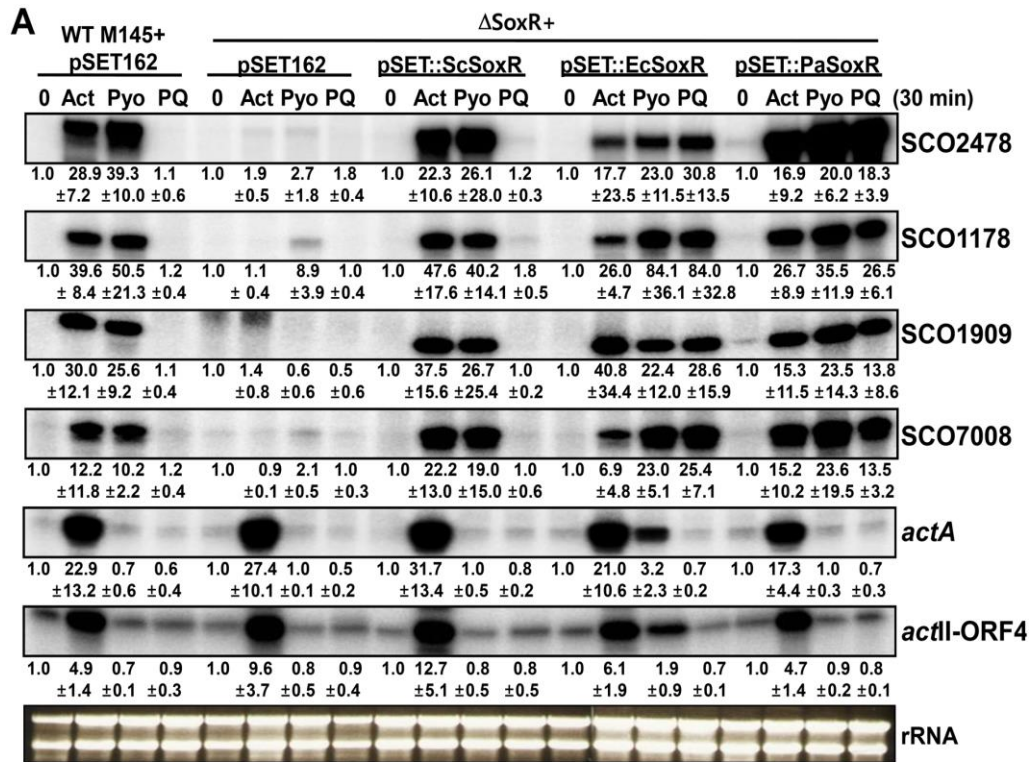


Fig. III-11. Differential activation of ScSoxR, EcSoxR, and PaSoxR expressed in *S. coelicolor* or in *E. coli*. (A) Activation profile in *S. coelicolor* cell background, genes for ScSoxR, EcSoxR, and PaSoxR were cloned in the pSET-152-derived integration vector pSET162 and introduced into the $\Delta soxR$ mutant strain of *S. coelicolor*. Since all these SoxRs share similar recognition sequences, we monitored the amount of SoxR target gene transcripts in *S. coelicolor*, as indicators of SoxR activation. Actinorhodin (Act; 500 nM), pyocyanin (Pyo; 100 μ M), or paraquat (PQ; 200 μ M) were added to exponentially growing *S. coelicolor* cells containing pSET162 vector, pSET162-ScSoxR, pSET162-EcSoxR, or pSET162-PaSoxR integrated at the *att* site in the chromosome. Gene-specific probes for SoxR targets (SCO2478, SCO1178, SCO1909, and SCO7008) were used for S1 mapping. As a control, transcripts known to be induced by actinorhodin (*actA* and *actII-ORF4*) were also measured. Relative expression levels were obtained from at least three independent experiments and are presented at the bottom of each dataset. (B) Activation profile in the *E. coli* cell background, genes for ScSoxR, EcSoxR, and PaSoxR were cloned in the multi-copy pTac4 plasmid. The recombinant plasmids were introduced into a $\Delta soxR$ *E. coli* GC4468 strain that contains the *soxSp*-driven β -galactosidase (*LacZ*) reporter gene in the chromosome. The transformed cells were grown in LB to early exponential phase ($OD_{600} \sim 0.2$) and either were left untreated or were treated with 200 μ M of PQ or 50 μ M of PMS for 60 min, followed by β -galactosidase activity assay. The mean values of activity in Miller units were obtained from three independent experiments. For each transformant, the induction fold relative to untreated level is indicated on top of graphic bar.

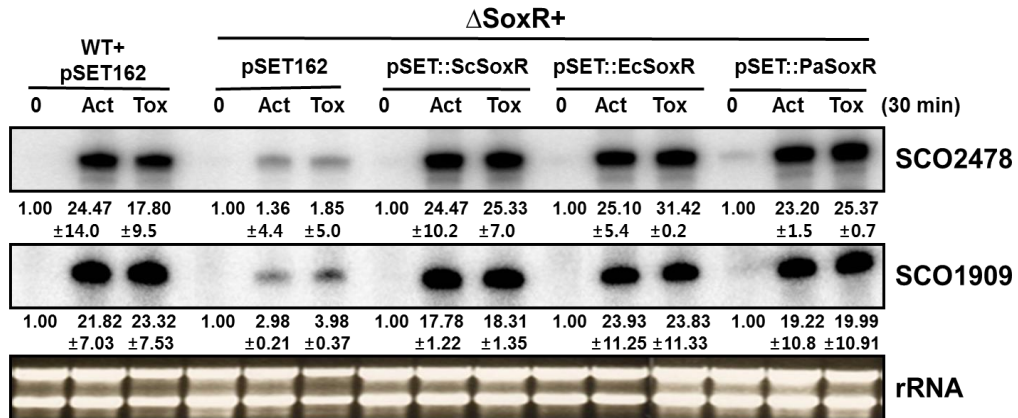


Fig. III-12. Induction of SoxR target genes by toxoflavin. *S. coelicolor* strains that contain genes for ScSoxR, EcSoxR, and PaSoxR were treated with toxoflavin (20 μ M) produced by *Burkholderia glumae* or with actinorhodin for 30 min as described in Fig. III-11.a. Transcripts from SoxR target genes (SCO2478 and SCO1909) were analyzed by S1 nuclease mapping. The relative expression values were calculated from three independent experiments.

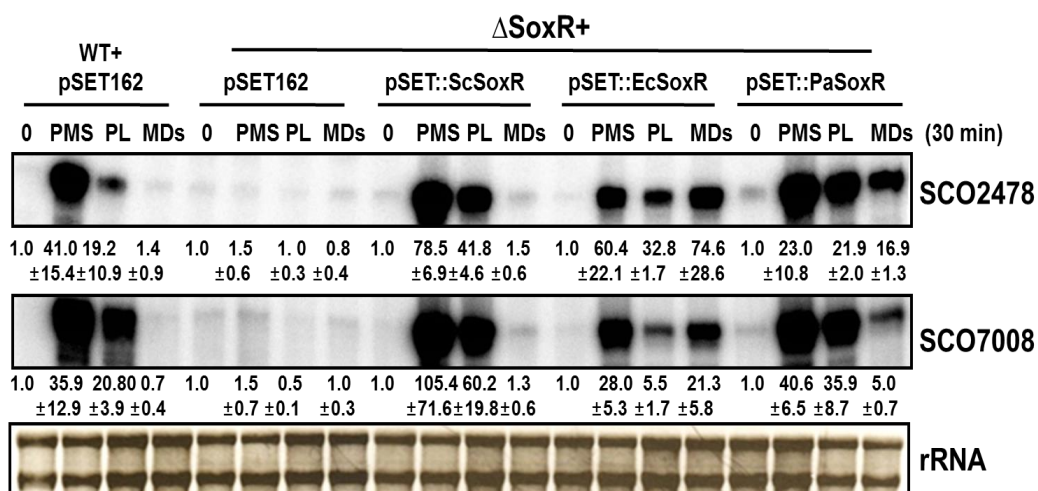


Fig. III-13. Induction of SoxR target genes by plumbagin (PL) and menadione sodium-bisulfite (MDs). Experiments were done as described in Fig. III-12 and Fig. III-11.a, except that plumbagin (25 μ M) or menadione sodium sulfite (350 μ M) were treated along with PMS (50 μ M) for 30 min. The relative expression values were calculated from three independent experiments.

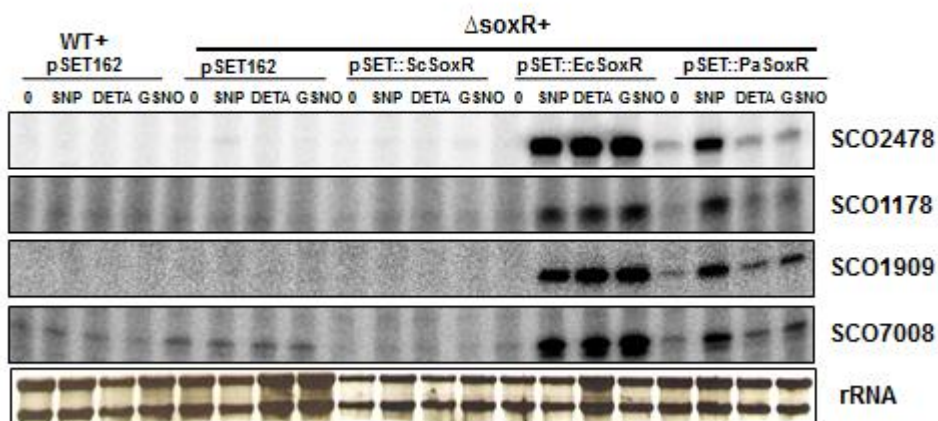


Fig. III-14. Induction of SoxR target genes by NO-generators. Experiments were done as described in Fig. III-12 and Fig. III-11.a, except that cells were treated for 30 min with NO-generating compounds: sodium nitroprusside (SNP; 1 mM), diethylenetriamine nitric oxide adduct (DETA-NO; 500 μ M), and S-nitrosoglutathione (GSNO; 300 μ M).

III.2.5. Time course of the activation of EcSoxR and PaSoxR by paraquat

Even though paraquat activates EcSoxR and PaSoxR, the extent of activation varies depending on experimental conditions. For example, paraquat activated PaSoxR in *P. aeruginosa*, but not as much as pyocyanin and PMS did (Fig. III-10). It activated PaSoxR in *S. coelicolor* cell background as well as other RACs did (Fig. III-11.a), whereas it did so slightly less effectively in an *E. coli* cell background (Fig. III-11.b). This variable effect may arise from the relatively poor action of paraquat as a direct oxidant. We therefore examined whether there are any differences in the kinetics of EcSoxR and PaSoxR activation by paraquat in their native cell backgrounds. We found that whereas paraquat activated EcSoxR to its maximal level within 2 min of treatment, it activated PaSoxR more slowly, reaching the maximal level only after 40 min (Fig. III-15). This difference explains the variable results obtained in different labs with different cell strains, culture conditions, and treatment protocols. Even with EcSoxR, which is effectively activated by paraquat, the kinetic experiments exhibited a large experimental fluctuation unless the treatment parameters such as duration and extent of aeration were standardized. The results in Fig. III-15, also implies that SoxRs with similar redox potential values can exhibit different responses to a chemical, due to differences that affect the kinetics of the redox reaction. Menadione bisulfite reacted more slowly with PaSoxR than with EcSoxR, as observed for paraquat (Fig. III-16).

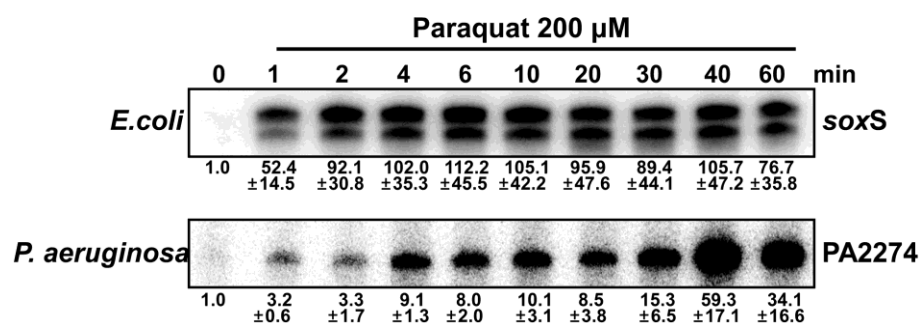


Fig. III-15. Time course of the activation of EcSoxR and PaSoxR by paraquat. Exponentially grown *E. coli* or *P. aeruginosa* wild type cells ($OD_{600} \sim 0.4$ to 0.5) were treated with 200μ M paraquat. At intervals (1 to 60 min), RNA samples were harvested from each culture, and the amount of SoxR target transcripts was analyzed by S1 mapping for *E. coli* (*soxS*) and *P. aeruginosa* (PA2274). Relative expression levels were obtained from at least three independent experiments and are presented at the bottom of each data set.

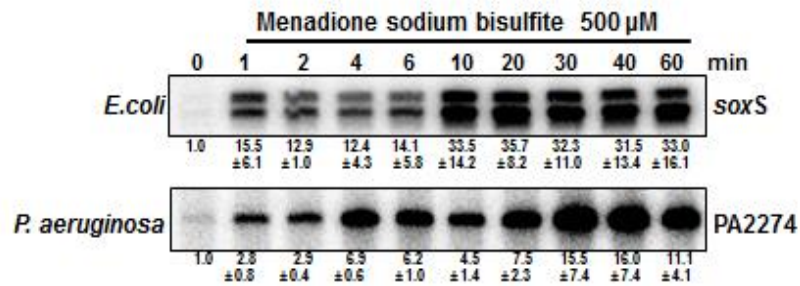


Fig. III-16. Time course of the activation of EcSoxR and PaSoxR by menadione sodium bisulfite. Exponentially grown wild type cells ($OD_{600} \sim 0.4$ to 0.5) were treated with $500 \mu\text{M}$ menadione bisulfite. At intervals (1 to 60 min) the amount of SoxR target transcripts was analyzed by S1 mapping for *E. coli* (*soxS*) and *P. aeruginosa* (PA2274). Relative expression levels were obtained from two independent experiments and are presented at the bottom of each data set.

III.2.6. In vivo redox status of [2Fe-2S] cluster of SoxRs following oxidant treatment

Whether the level of SoxR target gene transcripts indeed reflects the redox status of SoxR protein has not been examined for ScSoxR. Therefore, we monitored the redox status of ScSoxR overproduced in *E. coli* (XA90), by measuring X-band EPR spectra of the [2Fe-2S] cluster in the whole cell population at 15 K. EcSoxR and PaSoxR were measured in parallel for comparison. We observed that ScSoxR overproduced in an untreated cell sample demonstrated the characteristic spectral pattern of the reduced [2Fe-2S]¹⁺ cluster. The cluster was oxidized to its EPR-silent state when the cells were treated with PMS (Fig. III-17.a). In contrast, treatment with PQ did not diminish the signal, confirming that the inability of PQ to activate ScSoxR is indeed due to its inability to oxidize the [2Fe-2S]¹⁺ cluster of ScSoxR. EcSoxR overproduced in untreated *E. coli* cells gave rise to the characteristic EPR spectra reported previously (Fig. III-17.b) (Gu & Imlay, 2011; Gaudu *et al.*, 1997; Ding & Dimple, 1997). The spectra disappeared upon treatment with PQ and PMS. The EPR spectrum of PaSoxR was also similar to that of EcSoxR in untreated cells. PMS always silenced the spectral peaks, whereas the effect of PQ was somewhat variable. Data representative of four independent experiments is shown in Fig. III-17.c, demonstrating that PQ was partially effective in oxidizing the [2Fe-2S] cluster. This partial effect may lie behind the sub-maximal induction of PaSoxR by PQ in the *E. coli* cellular background (Fig. III-11.b) and may be the result of a slow reaction as implied from observations in Fig. III-12, the EPR results thus correlate with the measurements of SoxR target RNAs as indicators of SoxR activation.

III.2.7. Measurement of Redox Potential of ScSoxR

As an initial effort to find the mechanism behind the restricted reactivity of ScSoxR toward RACs, we set out to determine its redox potential by titration with sodium dithionite in the presence of the redox mediator safranin O (Massey, 1991), as described in Experimental procedures. For this purpose, ScSoxR and EcSoxR proteins were purified from *E. coli* and were resuspended to 10 μ M each in anaerobically prepared buffer. The UV-VIS absorption spectrum of air-oxidized ScSoxR indicated characteristic [2Fe-2S] peaks which disappeared upon the addition of sodium dithionite (Fig. III-18.a). The [2Fe-2S] cluster of ScSoxR was reduced by adding varying amounts of sodium dithionite in the presence of safranin O (5 μ M) at 29°C in anaerobic chamber. The redox potential of each solution was measured with a platinum and Ag/AgCl electrode (HACH-MTC101-1) and the redox status of SoxR was determined in the same solution by measurement of absorbance at 415 nm. Plots of the fraction of oxidized SoxR versus the redox potential (mV) of the solution revealed that the mid-point reduction potential of ScSoxR is -187 ± 10 mV (Fig. III-18). This value is about 100 mV higher than the estimated redox potential of EcSoxR (-287 ± 4 mV), which was measured in parallel. The value for EcSoxR is close to what was reported already [-285 ± 10 mV, (Ding *et al.*, 1996; Gaudu & Weiss, 1996)].

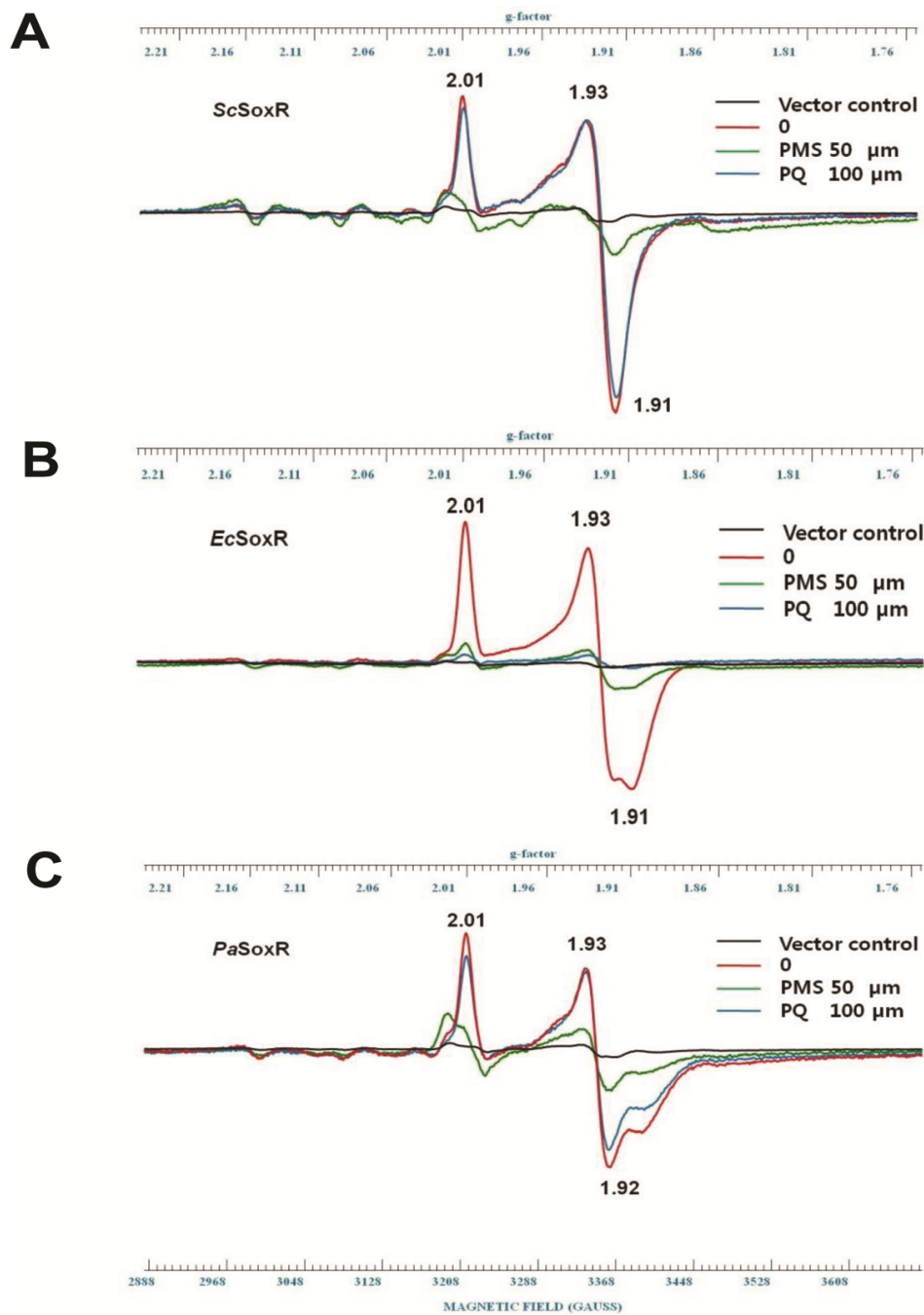


Fig. III-17. Whole cell EPR analysis of overexpressed SoxRs in *E. coli*. The redox status of the [2Fe-2S] clusters in ScSoxR, EcSoxR, and PaSoxR, which were overproduced in *E. coli*, was measured by EPR. The pTac4-based recombinant plasmids used in Fig. III-11.b, were introduced into *E. coli* XA90 cells. Each transformant strain was grown aerobically in LB medium to an OD₆₀₀ of 0.2, when IPTG was added, followed by further incubation at 37°C for more than 2 h or more until OD₆₀₀ reached 0.8 to 1.0. Either PMS (50 µM) or PQ (100 µM) was then added, and cultures were further incubated at 37°C for 40 min with shaking. After washing and resuspension, intact cells were transferred to EPR tubes and quickly frozen on dry ice. EPR measurements were performed at 15 K as described in Experimental procedures. EPR spectra from ScSoxR (**A**), EcSoxR (**B**), and PaSoxR (**C**) following treatment with PMS (green line), PQ (blue), or none (red) are presented with g-values for representative peaks indicated. Control spectra (black) from cells with parental vector only were also included. Representative spectral data from four independent experiments for each SoxR species are shown.

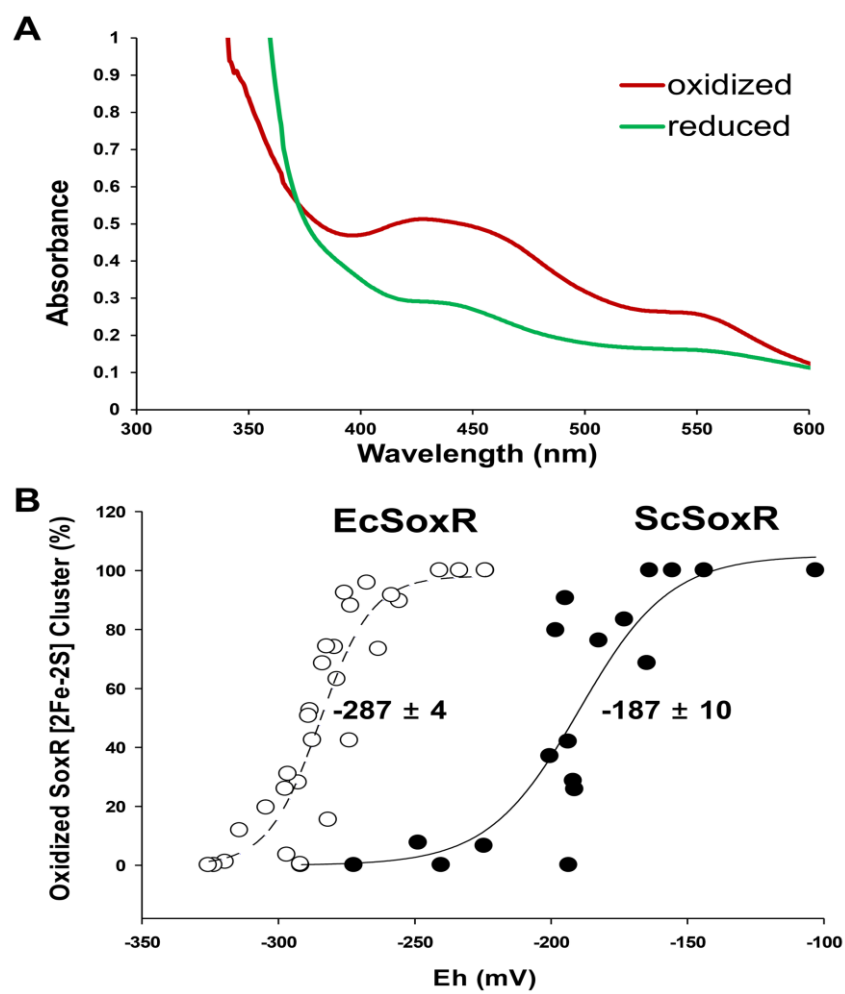


Fig. III-18. Redox titration of purified SoxR proteins. ScSoxR and EcSoxR proteins purified from *E. coli* were resuspended to 20 μ M each in 20 mM Tris-HCl (pH 7.8) containing 500 mM KCl, 10% glycerol, and 1 mM DTT in stoppered cuvettes. **(A)** Absorption spectra of oxidized and reduced ScSoxR were measured with UV-visible spectrophotometer. **(B)** The [2Fe-2S] cluster of the proteins were reduced in the presence of the redox mediator safranin O (5 μ M) at 25°C by adding different amounts of sodium dithionite in anaerobic chamber. The redox potential of the solution was measured with a platinum and Ag/AgCl electrode (HACH-MTC101-1), and the amount of oxidized SoxR in the same solution was measured by taking spectrophotometric absorbance at 415 nm as described in Experimental procedures. Percent fraction of oxidized SoxR (y-axis) was plotted against redox potential (Eh in mV) of the solution. The mid-point reduction potential of ScSoxR and EcSoxR was estimated to be -187 ± 10 and -287 ± 4 mV, respectively. Data shown here are representative of three independent experiments.

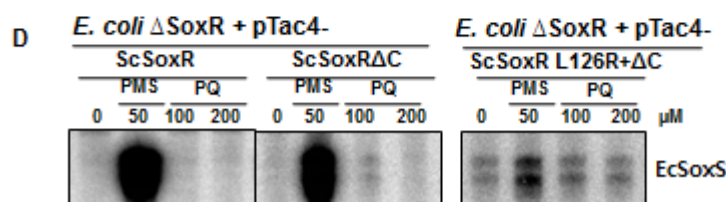
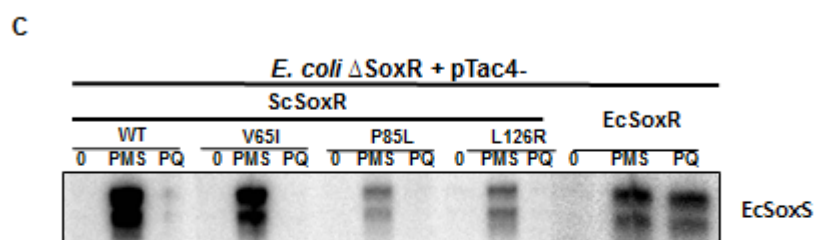
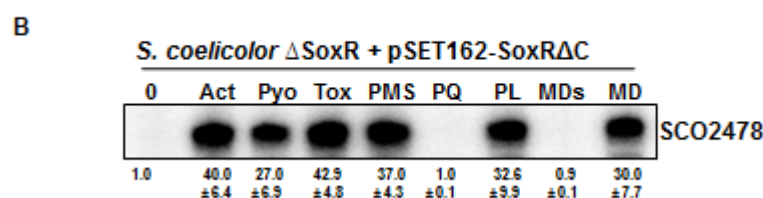
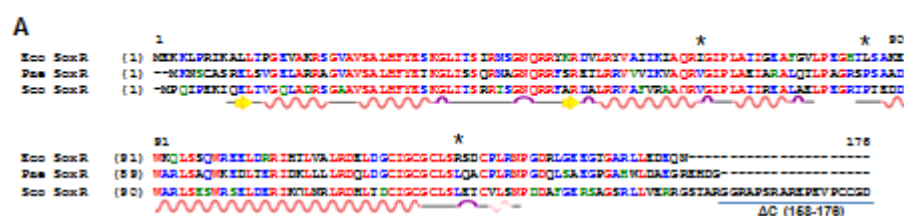


Fig. III-19. Mutations in specific residues of *S. coelicolor* SoxR. (A) Sequence comparison of SoxR homologs of *E. coli*, *P. aeruginosa* and *S. coelicolor*. The mutated residues are marked by asterisks (*) and underlined (Δ C). **(B)** Effect of C-terminal truncation of ScSoxR on reactivity in *S. coelicolor* host cell. The truncated gene for ScSoxR that lacks the C-terminal 18 aa from residue 158 to 175 (Δ C) was cloned in the integration vector pSET162 and introduced into the chromosome of *S. coelicolor* Δ soxR mutant. Cells were grown to OD₆₀₀ of ~ 0.4 to 0.5, and were treated with Act (200 nM), Pyo (10 μ M), Tox (20 μ M), PMS (50 μ M), PQ (200 μ M), PL (25 μ M), MDs (500 μ M), or MD (350 μ M) for 30 min. The amount of SoxR target (SCO2478) mRNA was then analyzed by S1 mapping. Relative expression levels were obtained from two independent experiments and are presented at the bottom of each data set. **(C)** Effect of substitution mutations on the reactivity of ScSoxR. Either wild type or mutated genes (V65I, P85L, or L126R) for ScSoxR or wild type EcSoxR were cloned in the multi-copy pTac4 plasmid. The recombinant plasmids were introduced into Δ soxR *E. coli* GC4468 strain that contains the soxSp-lacZ reporter. Exponentially grown cells (OD₆₀₀ ~ 0.4 to 0.5) were treated with PMS (50 μ M) or PQ (100 μ M) for 30 min. The amount of soxS mRNA was analyzed by S1 mapping **(D)** Effect of L126 mutation in Δ C mutant of ScSoxR. L126R mutation was introduced to the C-terminally truncated ScSoxR gene and cloned in the pTac4 plasmid. *E. coli* Δ soxR cells transformed with pTac4-ScSoxR, pTac4-ScSoxR Δ C, or pTac-ScSoxRL126R+ Δ C were grown, treated with oxidants, and analyzed as in **(C)**.

III.3. Mutational analysis of *Streptomyces coelicolor* SoxR to define the regions required for redox active molecules sensing and transcriptional activation

III.3.1. Mutations in specific residues of *S. coelicolor* SoxR alters its specificity for redox-active molecules

Sequence alignment of SoxR proteins around Fe-S clusters from various groups of bacteria can be seen in Fig. III-20 and crystal structure of *E. coli* SoxR is depicted in Fig. III-21 showing a depth information about SoxR transcriptional mechanism. Around the Fe-S cluster of SoxR has a conserved residue among gram-negative bacteria as well as some have in gram-positive bacteria. Sheplok *et al*, 2013 reported that there are three amino acid RSD motif that are conserved among gram negative bacteria and it is responsible for superoxide dependent gene regulation. (Singh *et al.*, 2013) reported that this residue is not responsible for superoxide dependent gene regulation in *S. coelicolor* and for looking into detailed amino acid residue around Fe-S cluster region, we made SWAP constructs with N-terminal of ScSoxR and C-terminal of EcSoxR. SWAP1 consists of ScSoxR V1 to S125 and EcSoxR R127 to N154 and SWAP2 consists of ScSoxR V1 to C129 and EcSoxR P131 to N154. ScSoxR truncated consists of V1 to R158 of ScSoxR (Fig. III-22). These constructs were then cloned into pTac4 vector. The resulting plasmid vector then transformed into *E. coli* soxR mutant. The transcriptional assays by S1 mapping described in (Fig. III-22) demonstrated that SWAP1 is sensitive towards all types of drugs such as PMS, PQ and MDs with varying degree of induction fold while SWAP2 and ScSoxR truncated sensitive by only PMS. Whereas, SWAP1 contains RSD motif which is conserved in family enterobacteriaceae. These result concluded that somehow RSD motif and some extended C-terminal region of *E. coli* SoxR might be responsible for sensing toward PQ and MDs.

The activation profiles for *S. coelicolor* SoxR (responsive to PMS but not PQ) were reminiscent of *E. coli* SoxR mutant proteins that were reported several years ago (Chander *et al.*, 2003; Chander & Demple, 2004). Further, elaborating on this experiment we made some single mutation by site directed mutagenesis within and around the Fe-S cluster region. For this purpose GC4468 soxR mutant background containing pTac4 vector with L126R, V130P and double mutant L126R+V130P ScSoxR truncated from *S. coelicolor* were grown in LB liquid media at OD₆₀₀ of 0.2-0.3 and thereafter IPTG treatment in same culture media for 1 hr, and then PMS (25 μ M), PQ (200 μ M) and MDs (500 μ M) were added and treated for 30 min before harvesting the cells. S1 mapping analysis demonstrated that in GC4468 soxR mutant background containing pTac4 vector with L126R, V130P and double mutant L126R+V130P ScSoxR truncated, PMS, PQ and MDs 500 μ M activated SoxS regulon and we found very interesting results (Fig. III-24). L126R did not responded to PQ and MDs while PMS activated it slightly, V130P showed almost similar results with L126R but PMS activated it more comparatively whereas double mutant L126R+V130P ScSoxR truncated behaving as Ec SoxR, responded to all redox compounds. These results show that this two amino acid arginine and proline in EcSoxR might be involved in superoxide gene regulation and that's why ScSoxR does not responds to superoxide.

We then exploited a *LacZ* reporter system in *E. coli* to monitor activation behavior of each mutant SoxRs species in another identical cellular background. Each mutant soxR gene was cloned in the pTac4 vector and was introduced into an *E. coli* soxR mutant that harbors a soxS promoter::*lacZ* fusion gene. Transformed cells were grown to exponential phase and treated with either 25 μ M PMS or 200 μ M PQ and 500 μ M MDs for 1 h. *LacZ* activity was then measured. Fig.III-25, showing that ScSoxR truncated L126R+V130P

and SWAP-1 were effectively induced by both PQ and PMS, whereas ScSoxR truncated, ScSoxR truncated V130P and ScSoxR truncated L126R were induced only by PMS. The absolute value of LacZ activity was relatively low in ScSoxR-containing *E. coli* strain. Thus, the activation profiles of mutant SoxR species that had been observed in the *S. coelicolor* cellular environment were reproduced in the *E. coli* background.

In continuation, we further examined the effect of superoxide generators such as Phenazine methosulfate (PMS), Paraquet (PQ) and Menadione sodium-bisulphate (MDs) in *S. coelicolor* M145 SoxR mutant background. For this purpose *S. coelicolor* soxR mutant background containing pSET162 vector containing promoter of ScSoxR with ScSoxR truncated, SWAP-1, ScSoxR truncated L126R, ScSoxR truncated V130P and double mutant ScSoxR truncated L126R+V130P from *S. coelicolor* were grown to mid-exponential phase (OD₆₀₀ of 0.4–0.5) in YEME liquid medium, and then PMS (25 µM), PQ (200 µM) and MDs (500 µM) were added and treated for 30 min before harvesting the cells. S1 mapping analysis demonstrated that in ScSoxR truncated and ScSoxR truncated V130P behave in a similar manner as M145 wild type and not responded to PQ, ScSoxR truncated L126R showed high expression by PMS but PQ and MDs low in comparisons to PMS, ScSoxR truncated L126R+V130P (Fig. III-26) showed high expression to all chemicals as well as no treatment suggesting that in *S. coelicolor* background this mutation behave as oxidized form of SoxR that's why there is consistent expression.

The overall result demonstrated that ScSoxR truncated L126R+V130P showing that superoxide sensing because leucine and valine is a hydrophobic (water-hating and usually internal) amino acid. As a result, the structure of the protein might change due to leucine and valine, desire not to be around

water. So yes, the position of the amino acid would change in terms of its position on the outside or inside of the protein. The desire of leucine and valine to not be in a water environment could disrupt the secondary, tertiary and quaternary structure (if any) of the protein whereas arginine and proline are hydrophilic (water loving and usually found on the outside of the protein) and react with PQ which is also water soluble and changes redox potential of SoxR and transcribes.

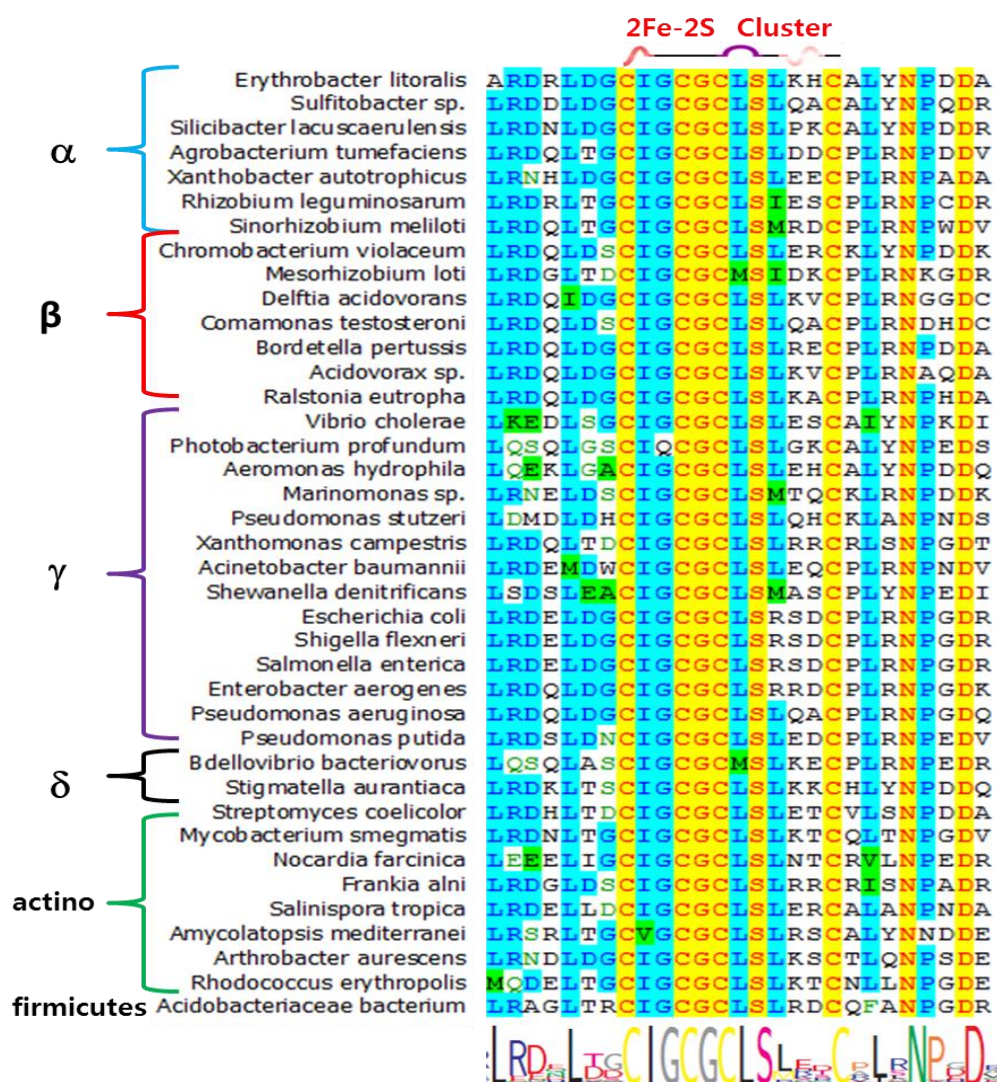


Fig. III-20. Sequence alignment of SoxRs. Based on the alignment of SoxR proteins within and around [2Fe-2S] cluster from selected group of bacteria from α, β, γ, δ, actinomycetes and firmicutes. An alignment was generated with Vector NTI. [2Fe-2S] cluster are shown in yellow color and upper written in [2Fe-2S] cluster. Species that contain the RSD motif in SoxR are in black. Bottom are the consensus logo of selected region of SoxRs protein.

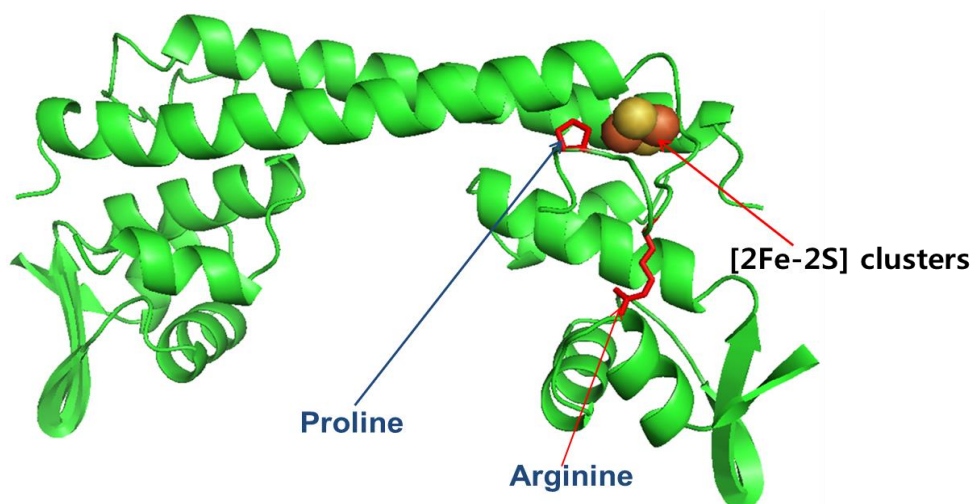


Fig. III-21. Crystal structure of *E.coli* SoxR locating arginine(R) within and proline (P) outside of [2Fe-2S] clusters. Location of key residues in *E. coli* SoxR. The structure of *E. coli* SoxR protein (Watanabe *et al.*, 2008). The amino- and carboxy-termini are indicated by N and C respectively, on one of the monomers. The [2Fe-2S] cluster in one monomer is labelled. Residues (R127, P131) identified as playing an important role in tuning the redox-reactivity of SoxR are shown.

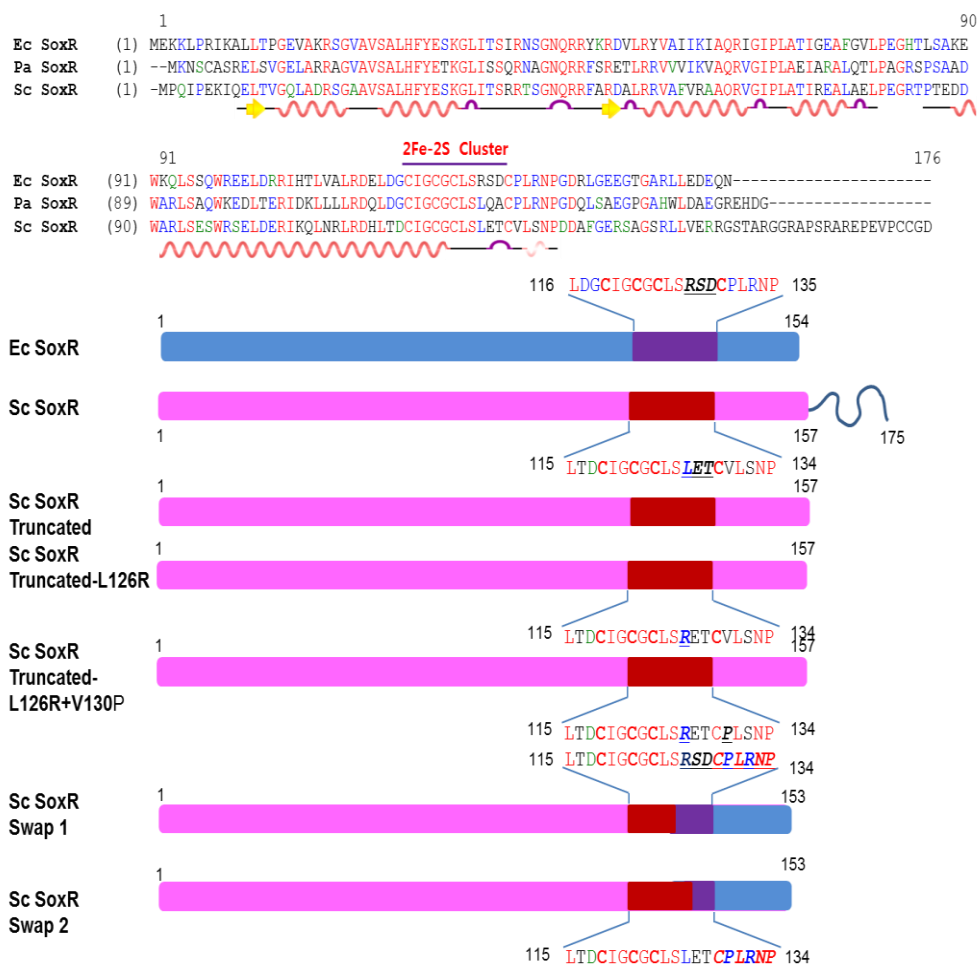


Fig. III-22. Sequence alignment of Sc, Ec ,Pa SoxR and schematic representations of mutant SoxR. Sequence comparison of SoxR homologs of *E. coli*, *P. aeruginosa* and *S. coelicolor* and various mutant and specific residue are represented in bold.

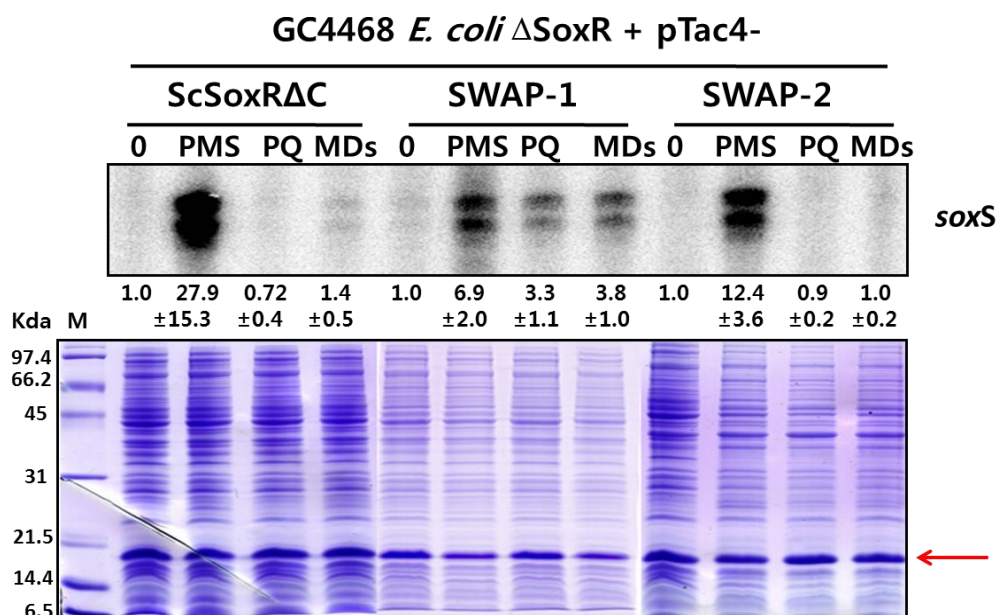


Fig. III-23. Effect of a swapping of *S.coelicolor* SoxR with *E.coli* SoxR.

The truncated gene for ScSoxR that lacks the C-terminal 18 aa from residue 158 to 175 and SWAP-1 that contain ScSoxR of V1-S125 and EcSoxR of R127-N154 and SWAP-2 ScSoxR of V1-C129 EcSoxR of P131-N154 and was cloned in the pTac4 plasmid and introduced into the *E. coli* Δ soxR strain. Exponentially grown cells ($OD_{600} \sim 0.4$ to 0.5) were treated with PMS ($25 \mu M$), PQ ($200 \mu M$) and MDs ($500 \mu M$) for 30 min and the amount of soxS mRNA was analyzed by S1 mapping, the relative expression values were calculated from three independent experiments. Bottom (SDS-PAGE figure): parallel checking of protein levels by analyzing on SDS-PAGE obtained from same cell culture by taking $200 \mu l$ cell sample with arrow indicating the expressed protein level.

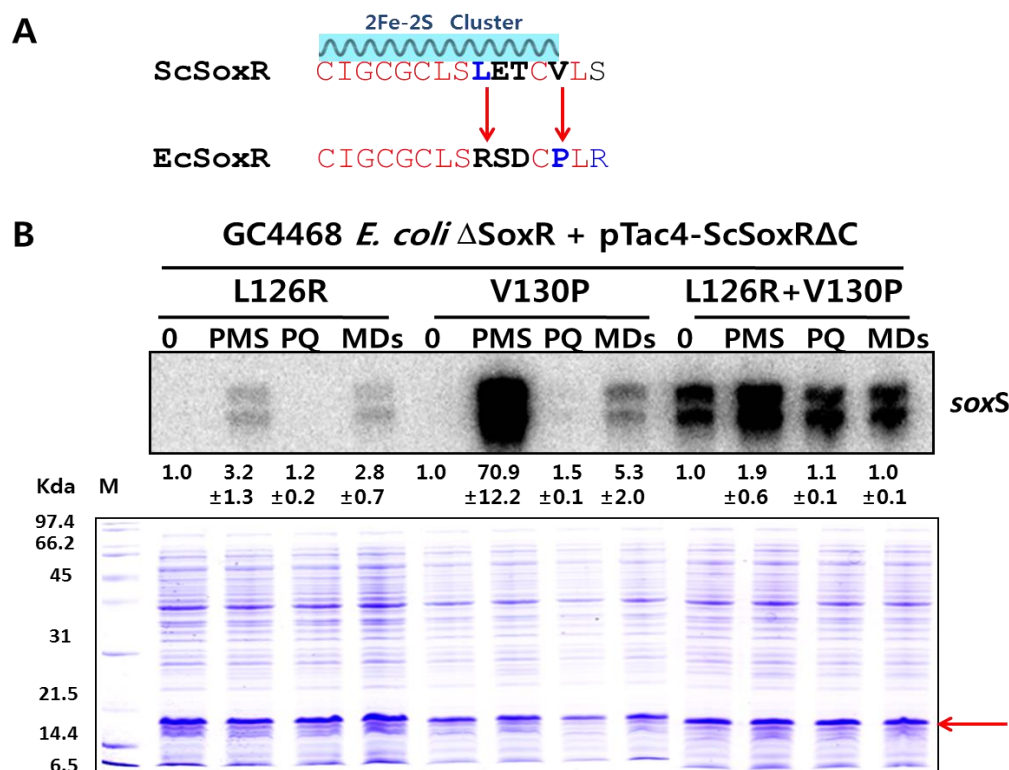


Fig. III-24. Mutations in specific residues of *S.coelicolor* SoxR and showing transcriptional level gene expression in *E.coli* Δ soxR background .

(A) Schematic representations of mutated region of ScSoxR and by arrow show the final mutated amino acid (B) Effect of substitution mutations on the reactivity of ScSoxR. Either wild type with truncated mutated genes (L126R, V130P and L126R+V130P) for ScSoxR were cloned in the multi-copy pTac4 plasmid. The recombinant plasmids were introduced into Δ soxR *E. coli* GC4468 strain that contains the soxSp-lacZ reporter. Exponentially grown cells ($OD_{600} \sim 0.4$ to 0.5) were treated with PMS ($25 \mu M$), PQ ($200 \mu M$) and MDs ($500 \mu M$) for 30 min. The amount of soxS mRNA was analyzed by S1 mapping. The relative expression values were calculated from three independent experiments. Bottom (SDS-PAGE figure): parallel checking of protein levels by analyzing on SDS-PAGE obtained from same cell culture by taking $200 \mu l$ cell sample with arrow indicating the expressed protein level.

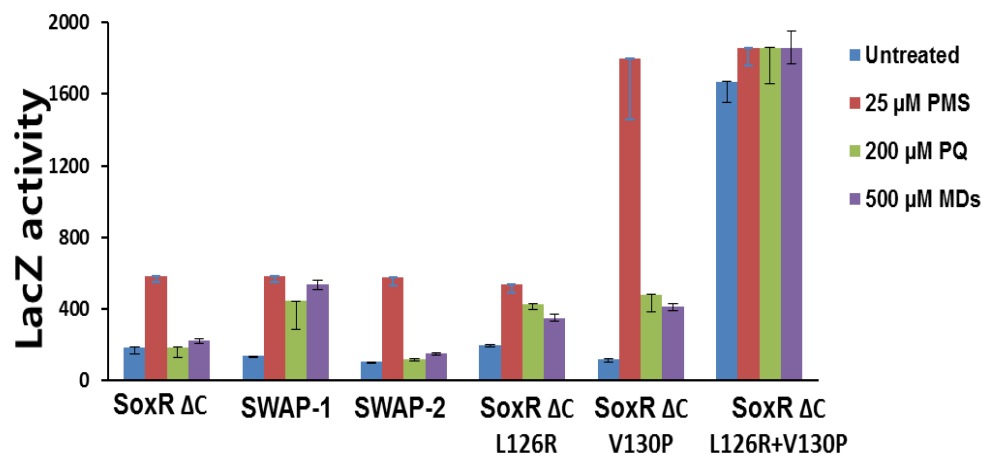


Fig. III-25. β -galactosidase (*LacZ*) reporter gene assay. Activation profile in the *E. coli* cell background, genes for ScSoxR Δ C, SWAP-1, SWAP-2, ScSoxR Δ C-L126R, ScSoxR Δ C-V130P and ScSoxR Δ C-L126R+V130P were cloned in the multi-copy pTac4 plasmid. The recombinant plasmids were introduced into a Δ soxR *E. coli* GC4468 strain that contains the soxSp-driven β -galactosidase (*LacZ*) reporter gene in the chromosome. The transformed cells were grown in LB to early exponential phase ($OD_{600} \sim 0.2$) and either were left untreated or were treated with 25 μ M of PMS, 200 μ M of PQ and 500 μ M of Mds for 60 min, followed by β -galactosidase activity assay.

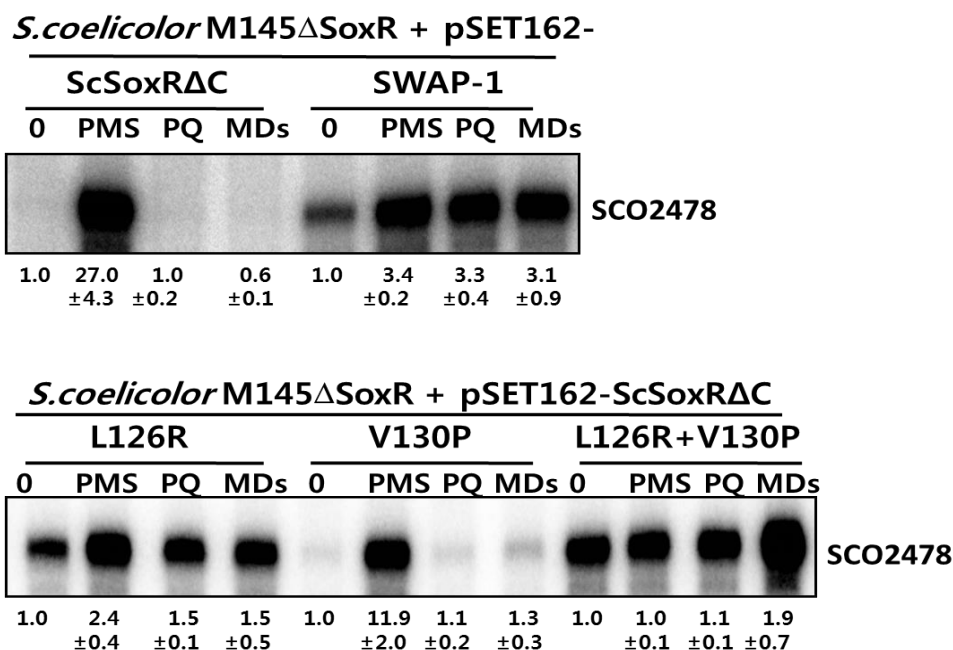


Fig. III-26. Mutations in specific residues of *S.coelicolor* SoxR and showing transcriptional level gene expression in *S.coelicolor* Δ soxR background .

Effect of substitution mutations on the reactivity of ScSoxR. Either wild type with truncated, Swap-1 and ScSoxR Δ C (L126R, V130P and L126R+V130P) for ScSoxR were cloned in the pSET-152-derived integration vector pSET162 and introduced into the Δ soxR mutant strain of *S. coelicolor*. We monitored the amount of SoxR target gene transcripts in *S. coelicolor*, as indicators of SoxR activation. 25 μ M of PMS, 200 μ M of PQ and 500 μ M of MDs were added to exponentially growing *S. coelicolor* cells containing pSET162 vector, pSET162-ScSoxR Δ C, pSET162-SWAP-1, pSET162-ScSoxR Δ C-L126R, pSET162-ScSoxR Δ C-V130P and pSET162-ScSoxR Δ C-L126R+V130P integrated at the att site in the chromosome. Gene-specific probes for SoxR target SCO2478 was used for S1 mapping. Relative expression levels were obtained from at least three independent experiments and are presented at the bottom of each dataset.

III.3.2. In vivo redox status of [2Fe-2S] cluster of *S. coelicolor* SoxR, ScSoxR-L126R and SWAP-1 without treatment

Whether the level of ScSoxR, ScSoxR-L126R and SWAP-1 and transcripts indeed reflects the redox status of SoxR protein has not been examined for ScSoxR-L126R and SWAP-1. Therefore, we monitored the redox status of ScSoxR, ScSoxR-L126R and SWAP-1 overproduced in *E. coli* (XA90), by measuring X-band EPR spectra of the [2Fe-2S] cluster in the whole cell population at 15 K. ScSoxR was measured in parallel for comparison with ScSoxR-L126R and SWAP-1. We observed that ScSoxR overproduced in an untreated cell sample demonstrated the characteristic spectral pattern of the reduced [2Fe-2S] cluster. ScSoxR-L126R and SWAP-1 overproduced in untreated *E. coli* cells gave rise to the characteristic EPR spectra (Fig. III-27) with variation to ScSoxR. This showed that they looked like oxidized form of protein or might be somehow [2Fe-2S] the clusters are destabilized.

III.3.3. Mutations in specific residues of *E.coli* SoxR alters its specificity toward paraquat (PQ)

We then examined the specific mutation of some residue around [2Fe-2S] cluster in *E. coli* SoxR which might possibly change their behavior like ScSoxR. For this purpose we mutated EcSoxR-R127L, P131V and double site directed mutation R127L+P131V and constructed in pTac4 plasmid vector. Then, GC4468 soxR mutant background containing pTac4 vector with R127L, P131V and R127L+P131V and were grown to LB liquid media at OD₆₀₀ of 0.2–0.3 and thereafter IPTG treatment in same culture media for 1 hr, and then PMS (25 µM), PQ (200 µM) and MDs (500 µM) were added and treated for 30 min before harvesting the cells. S1 mapping analysis demonstrated that in GC4468 soxR mutant background containing pTac4 vector with R127L and P131V, are PMS, PQ and MDs 500µM activated SoxS regulon and we found

that double mutation R127L+P131V are behaving like ScSoxR (Fig. III-28). For clear confirmation of these results, we further elaborated this experiment, for this purpose *S.coelicolor* soxR mutant background containing pSET162 vector containing promoter of ScSoxR with EcSoxR-R127L. EcSoxR-P131V and EcSoxR-R127L+P131V from *S. coelicolor* were grown to mid-exponential phase (OD₆₀₀ of 0.4–0.5) in YEME liquid medium, and then PMS (25µM), PQ (200 µM) and MDs (500 µM) were added and treated for 30 min before harvesting the cells. S1 mapping analysis demonstrated that in EcSoxR, EcSoxR-R127L and EcSoxR-P131V behaved in a similar pattern whereas double mutant looks like ScSoxR (Fig. III-29).

The overall result demonstrated that EcSoxR-R127L+P131V were unable to show superoxide sensing and these two amino acid are indeed responsible for superoxide sensing in *E. coli*.

III.3.4. Mutations in *M. smegmatis* SoxR around Fe-S cluster specific residue

We further studied to check whether these mutations in specific residues of SoxR that alters, its specificity for redox-active molecules mechanism is also true for other actinomycets. For this, we selected *Mycobacterium smegmatis*, which is an acid-fast bacterial species in the phylum Actinobacteria and the genus *Mycobacterium*. *Mycobacterium* is generally considered a non-pathogenic microorganism; however, in some very rare cases, it may cause disease. *M. smegmatis* is commonly used in work on the mycobacterium species due to its being a "fast grower" and non-pathogenic.

For this purpose we constructed *M. smegmatis* soxR gene in pTac4 vector and cloned into an GC4468 *E. coli* soxR mutant background and selected some colony and were grown to LB liquid media at OD₆₀₀ of 0.2–0.3 and thereafter IPTG treatment in same culture media for 1 hr, and then PMS

(25 μ M), PQ (200 μ M) and MDs (500 μ M) were added and treated for 30 min before harvesting the cells. S1 mapping analysis demonstrated that in GC4468 *soxR* mutant background containing pTac4 vector with *M. smegmatis soxR*, PMS and MDs activated SoxS regulon and whereas superoxide generating compound PQ were unable to activate *soxS* transcript (Fig. III-30).

In continuation, we further examined the effect of then PMS, PQ and MDs in GC4468 *E. coli soxR* mutant background containing MsSoxR-L121R, MsSoxR-Q125P and double mutant MsSoxR-L121R+Q125P in Ptac4 vector and grow and RNA isolation as above. S1 mapping analysis demonstrated that in GC4468 *soxR* mutant background containing pTac4 vector with MsSoxR-L121R and MsSoxR-Q125P are slightly expressed by superoxide(PQ) whereas double mutant MsSoxR-L121R+Q125P, showing expression level of *soxS* in all chemical as well as untreated sample also(Fig. III-30).

We then exploited a *LacZ* reporter system in *E. coli* to monitor activation behavior of each mutant MsSoxRs species in another methodology with same strain background. Each mutant MsSoxR gene was cloned in the pTac4 vector and was introduced into an *E. coli soxR* mutant that harbors a *soxS* promoter::*lacZ* fusion gene. Transformed cells were grown to exponential phase and treated with either 25 μ M PMS or 200 μ M PQ and 500 μ M MDs for 1 h. *LacZ* activity was then measured. Fig. III-31 shows that MsSoxR is effectively induced by only PMS, whereas MsSoxR-L121R, MsSoxR-Q125P and double mutant MsSoxR-L121R+Q125P were induced by PMS, PQ and MDs. These results are consistent with S1 mapping analysis.

The results are not consistent with ScSoxR in case of single and double mutation of MsSoxR and make behave like EcSoxR. On the basis of these results, we conclude that these two amino acids those are conserved within [2Fe-2S] cluster region but are not directly responsible for activation of redox activity in all actinomycetes.

III.3.5. Slower differentiation and decreased production of antibiotics in Δ soxR mutants

When plated on solid R2YE, the Δ soxR mutant was delayed in forming aerial mycelia and pigmented antibiotics by about 2 to 6 days whereas Δ soxR Δ CL126R+V130P showing more delay growth than Δ soxR mutant strain (Fig. III-32). The amounts of sporulation as well as red and blue pigments were also reduced in the Δ soxR mutant and more in Δ soxR Δ CL126R+V130P strain. This phenomenon is reproducibly observed on R2YE plates but not much on other media such as SFM plates (Fig. III-32).

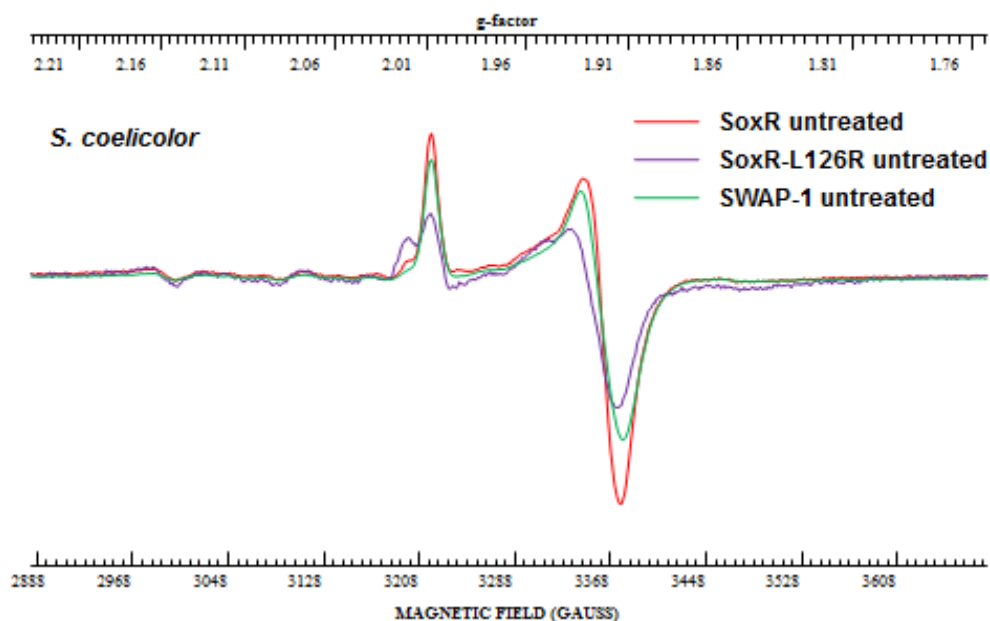


Fig. III-27. Whole cell EPR analysis of overexpressed SoxRs in *E. coli*.

The redox status of the [2Fe-2S] clusters in ScSoxR, ScSoxR-L126R, and SWAP-1, which were overproduced in *E. coli*, were measured by EPR. The pTac4-based recombinant plasmids used and were introduced into *E. coli* XA90 cells. Each transformant strain was grown aerobically in LB medium to an OD₆₀₀ of 0.2, when IPTG was added, followed by further incubation at 37°C for more than 2 h or more until OD₆₀₀ reached 0.8 to 1.0, and cultures were further incubated at 37°C for 40 min with shaking. After washing and resuspension, intact cells were transferred to EPR tubes and quickly frozen on dry ice. EPR measurements were performed at 15 K as described in Experimental procedures. EPR spectra from ScSoxR, ScSoxR-L126R, and SWAP-1 are presented with g-values for representative peaks indicated. Representative spectral data from four independent experiments for each SoxR species are shown.

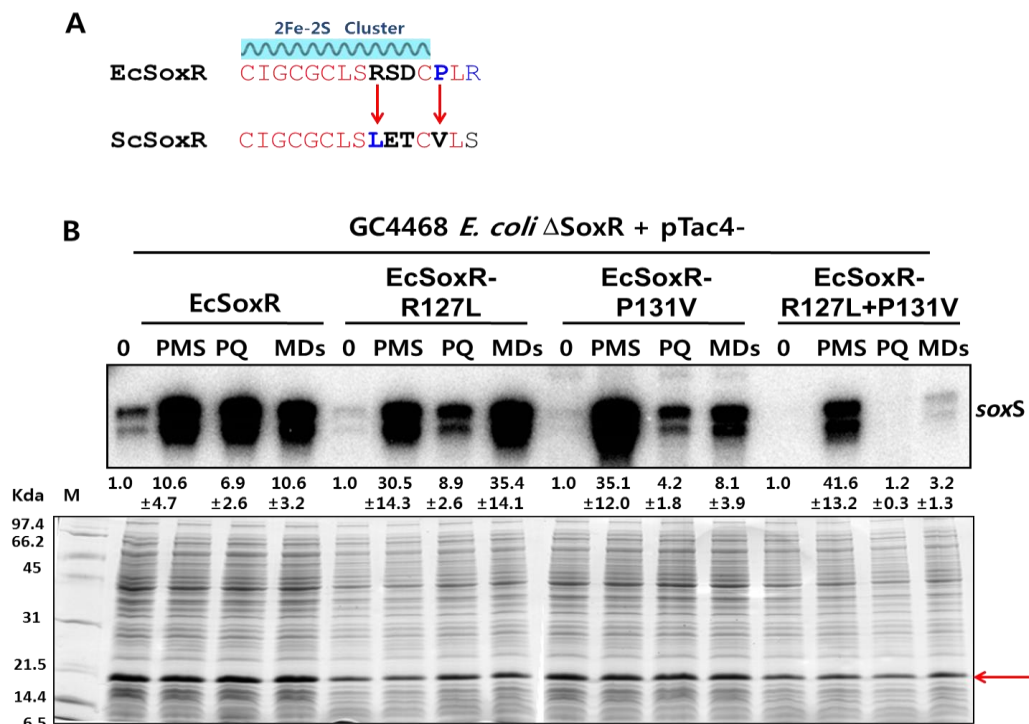


Fig. III-28. Mutations in specific residues of *E.coli* SoxR and showing transcript level gene expression in *E.coli* Δ soxR background .

(A) Schematic representations of mutated region of EcSoxR and by arrow show the final mutated amino acid (B) Effect of substitution mutations on the reactivity of EcSoxR. Either wild type with mutated genes (R127L, P131V and R127L+P131V) for EcSoxR were cloned in the multi-copy pTac4 plasmid. The recombinant plasmids were introduced into Δ soxR *E. coli* GC4468 strain that contains the soxSp-lacZ reporter. Exponentially grown cells ($OD_{600} \sim 0.4$ to 0.5) were treated with PMS ($25 \mu M$), PQ ($200 \mu M$) and MDs ($500 \mu M$) for 30 min. The amount of soxS mRNA was analyzed by S1 mapping. The relative expression values were calculated from three independent experiments. Bottom (SDS_PAGE figure): parallel checking of protein levels by analyzing on SDS-PAGE obtained from same cell culture by taking $200 \mu l$ cell sample with arrow indicating the expressed protein level.

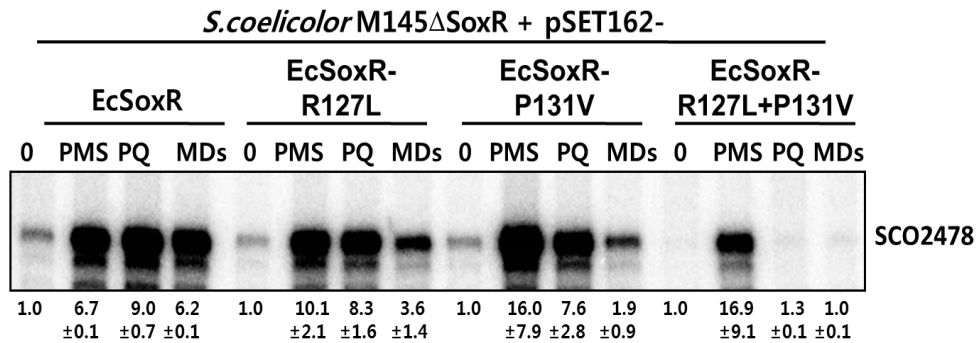


Fig. III-29. Mutations in specific residues of *E.coli* SoxR and showing transcriptional level gene expression in *S.coelicolor* Δ soxR background .

Effect of substitution mutations on the reactivity of EcSoxR. Either wild type or EcSoxR with mutated region (R127L, P131V and R127L+P131V) for EcSoxR were cloned in the pSET-152-derived integration vector pSET162 and introduced into the Δ soxR mutant strain of *S. coelicolor*. We monitored the amount of SoxR target gene transcripts in *S. coelicolor*, as indicators of SoxR activation. 25 μ M of PMS, 200 μ M of PQ and 500 μ M of Mds were added to exponentially growing *S. coelicolor* cells containing pSET162 vector, pSET162-EcSoxR, pSET162-EcSoxR-R127L, pSET162-EcSoxR-P131V and pSET162-EcSoxR- R127L+P131V integrated at the att site in the chromosome. Gene-specific probes for SoxR target SCO2478 was used for S1 mapping. Relative expression levels were obtained from at least three independent experiments and are presented at the bottom of each dataset.

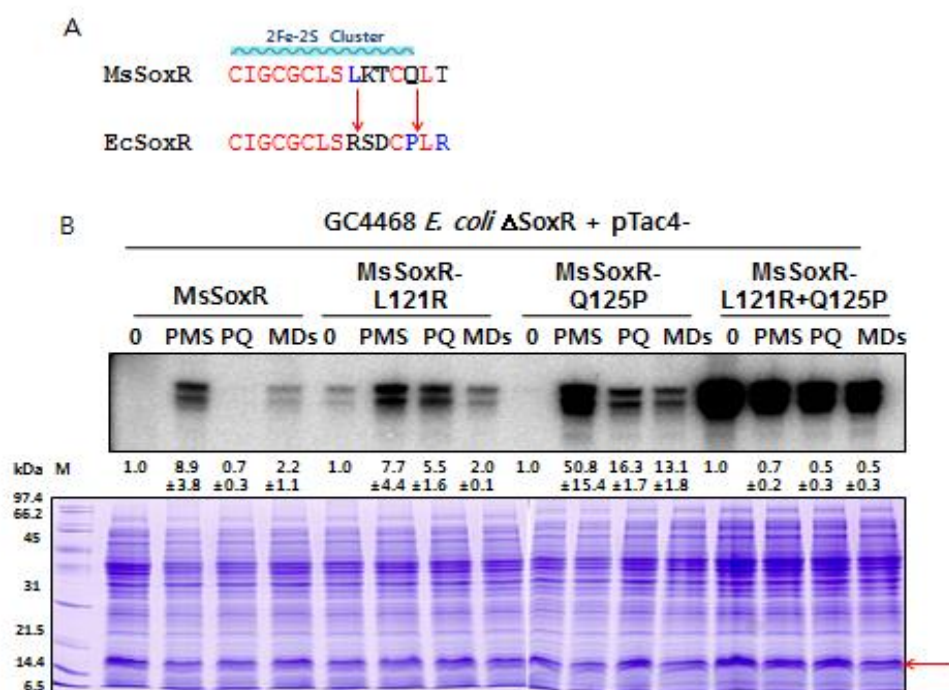


Fig. III-30. Mutations in specific residues of *M.smegmatis* SoxR.

(A) Schematic representations of mutated region of MsSoxR and by arrow show the final mutated amino acid **(B)** Effect of substitution mutations on the reactivity of MsSoxR. Either wild type or mutated genes (L121R, Q125P and L121R+Q125P) for MsSoxR were cloned in the multi-copy pTac4 plasmid. The recombinant plasmids were introduced into Δ soxR *E. coli* GC4468 strain that contains the soxSp-lacZ reporter. Exponentially grown cells ($OD_{600} \sim 0.4$ to 0.5) were treated with PMS (25 μ M) , PQ (200 μ M) and MDs (500 μ M) for 30 min. The amount of soxS mRNA was analyzed by S1 mapping. The relative expression values were calculated from three independent experiments. Bottom (SDS-PAGE figure): parallel checking of protein levels by analyzing on SDS-PAGE obtained from same cell culture by taking 200 μ l cell sample with arrow indicating the expressed protein level.

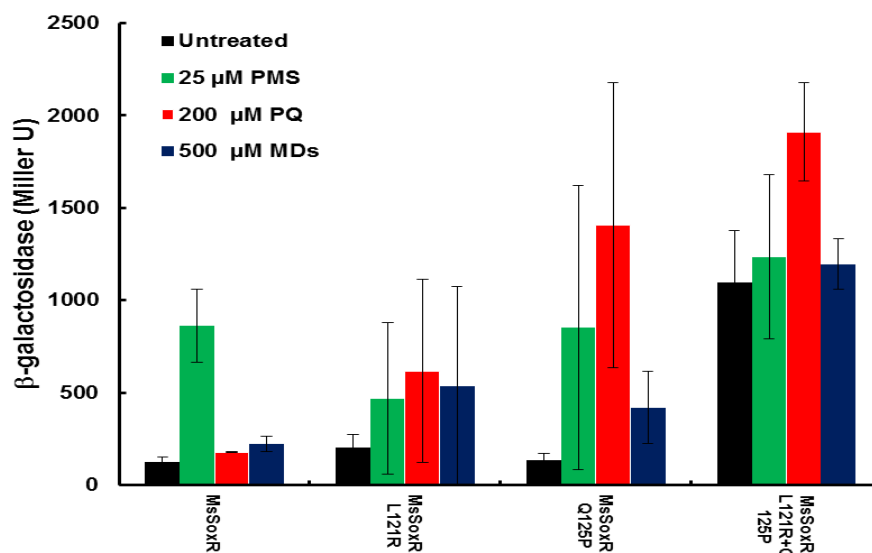


Fig. III-31. β -galactosidase (*LacZ*) reporter gene assay of *M. smegmatis* SoxR and their mutants. Activation profile in the *E. coli* cell background, genes for MsSoxR, MsSoxR-L121R, MsSoxR-Q125P and MsSoxR-L121R+Q125P were cloned in the multi-copy pTac4 plasmid. The recombinant plasmids were introduced into a Δ soxR *E. coli* GC4468 strain that contains the *soxSp*-driven β -galactosidase (*LacZ*) reporter gene in the chromosome. The transformed cells were grown in LB to early exponential phase ($OD_{600} \sim 0.2$) and either were left untreated or were treated with 25 μ M of PMS, 200 μ M of PQ and 500 μ M of MDs for 60 min, followed by β -galactosidase activity assay. The mean values of activity in Miller units were obtained from three independent experiments.

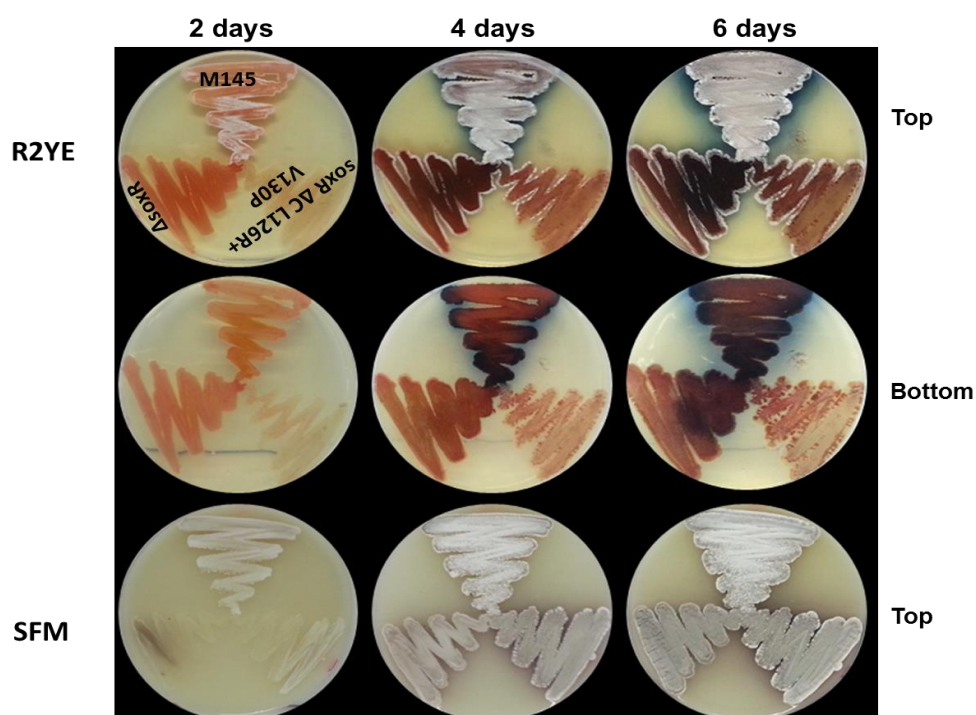


Fig. III-32. Differentiation and antibiotics-producing phenotypes of Δ soxR and soxR Δ C L126R+V130P mutant strains on the R2YE & SFM solid media. Differentiation progress of wild type (M145 with pSET162), Δ soxR with pSET162, Δ soxR complemented with pSET162:: soxR Δ C-L126R+V130P strains on R2YE and SFM solid plates. Formation of aerial mycelia, spores, and pigmented antibiotics was examined visually by taking photos at 2, 4, and 6 days after inoculation.

CHAPTER IV

DISCUSSION

In this work, we demonstrated that ScSoxR responded to a limited range of chemicals, whereas PaSoxR and EcSoxR responded to nearly all or all redox-active chemicals (RACs), respectively. The activation of target gene expression by ScSoxR in response to RAC correlated with the oxidation of its [2Fe-2S] cluster by the effector chemical, as shown by whole cell EPR analysis. We also found that ScSoxR confers adaptive protection to the cell against growth-inhibitory effect of inducing chemicals.

What features of ScSoxR, in comparison with other SoxRs, determine its selective behavior? Regarding its insensitivity to paraquat, I believe that the relatively high redox potential of ScSoxR (-187 mV) in comparison with those of EcSoxR (-285 mV;(Ding *et al.*, 1996; Gaudu & Weiss, 1996); this study) and PaSoxR (-290 mV; (Kobayashi & Tagawa, 2004) makes it less favorable to get oxidized by the weak oxidant paraquat (-440 mV; (Steckhan & Kuwana, 1974). One can wonder why PQ is able to oxidize SoxR if the reduction potential of SoxR is so much higher than that of PQ. There are two parts to this. First, the reduced PQ is quickly consumed by electron transfer to other, higher-potential acceptors-molecular oxygen or ubiquinone in the respiratory chain. Thus, the uphill electron transfer is pulled forward by the downhill nature of the subsequent electron transfers. Second, the reduction potentials do affect the rate of the first electron transfer. The more uphill this reaction is, the slower it will be. The reaction is more uphill for ScSoxR than for EcSoxR. In quantitative terms, electron transfer from -287 mV to -440 mV represents an uphill reaction of +3,528 cal, compared to +5,834 cal if the donor is at -187 mV. These positive free energies do not require that the reactions be slow; electron-transfer reactions with these positive free energies can nevertheless occur on very short time scales.

Electron transfer from EcSoxR (-287 mV) to paraquat (-440 mV) constitutes a ΔE° of -153 mV. Since $\Delta G^{\circ} = -nF\Delta E^{\circ}$, where for this reaction

$n=1$ electron and $F=23060$ cal/mole, ΔG° for this reaction $=+3528$ cal. Similarly, transfer from ScSoxR (-187 mV) to paraquat involves $\Delta G^\circ =+5834$ cal. The rate of electron transfer depends upon the energy of activation E_a with the relation $k=Ae^{-E_a/RT}$, where k is the rate constant, A is the frequency factor, R is gas constant, and T is the temperature in Kelvin. The ΔG° values almost certainly contribute to the energy of activation. By substituting these ΔG° values for E_a , it is calculated that the difference in rate of electron transfer will be a factor of 40. (This is also true if the E_a of the two reactions also includes any other constant term to which ΔG° of electron transfer must be added). That is, raising the SoxR potential by 100 mV will slow its oxidation rate by paraquat by a factor of 40. So it makes absolute sense that the redox potential difference will push PQ to oxidize EcSoxR much more quickly than ScSoxR.

Does a high energy of activation require that the reaction be slow? Diffusion-limited reactions in aqueous solution have second-order rate constants of $\sim 10^{10}$ M⁻¹s⁻¹. That is the fastest possible rate constant, representing successful electron transfer upon every encounter between redox partners in aqueous solution. An activation energy of +3528 cal would potentially slow this reaction by a factor of $e^{-E_a/RT}$, or 300-fold. Thus, if no other factors were involved, the rate constant might in principle be as high as 3×10^7 M⁻¹s⁻¹. This second-order rate equation can be used: $\ln[\text{SoxR}_t/\text{SoxR}_o] = -k[\text{PQ}]t$. Thus if there were 100 μM PQ²⁺ in the cell, this rate constant implies that SoxR could be oxidized with a half-time as short as 0.2 msec. Of course, there certainly will be other contributions to the activation energy, and orientation factors will also intervene, so the actual rate constant will be lower and the reaction will be substantially slower. However, simply because the ΔG° is +3,528 cal does not mean that the reaction must be too slow to activate the SoxR regulon.

The free energy difference between SoxR and paraquat will almost certainly constitute part of the energy of activation, and calculations indicate that the lower potential of EcSoxR could accelerate the reaction by as much as 40-fold relative to the higher potential of ScSoxR. Thus the potential difference is a compelling explanation for the different responsiveness of the SoxR proteins to paraquat. Of course, if there are structural differences that influence how easily an oxidant approaches the clusters, then this will have an additional effect.

As observed in our study, the redox potential cannot be the sole factor in determining selectivity, since menadione sodium bisulfite (MDs), which is a potent oxidant in terms of redox potential (-45 mV; (Hodnick & Sartorelli, 1997)), does not activate ScSoxR. The negative charge of MDs might impede the association of the compound with the redox area of SoxR. Therefore, the redox potential, which is an equilibrium value, could be a necessary factor in determining redox reaction, but not a sufficient factor. It can be hypothesized that the accessibility of the Fe-S center to reactive chemicals may differ among SoxRs, even though the [2Fe-2S] cluster is thought to be solvent-exposed, as determined for oxidized EcSoxR (Watanabe *et al.*, 2008). The sensitivity of PaSoxR toward paraquat saliently supports this proposal. The redox-cycling weak oxidant paraquat can oxidize both PaSoxR and EcSoxR, whose Fe-S clusters are of similar redox potential. However, this study shows that PaSoxR responds more slowly to paraquat than does EcSoxR. This reveals the contribution of kinetic factors, such as accessibility and reactivity, in determining the feasibility of redox reaction between SoxR and RACs. The different kinetics of the redox reactions most likely explains why different labs observe different sensitivity patterns for redox-cycling weak oxidants, if the experimental conditions and lab strains are not standardized. Then what determines the redox potential and kinetic reactivity of the [2Fe-2S] cluster in

each SoxR? More study will be needed to delineate which residues or structural features affect the redox potential and which affect kinetic parameters. However, the combined contribution of redox potential and kinetic parameters is reflected in the overall reactivity or oxidizability of individual SoxR proteins by particular compounds. Using target RNA analysis to monitor SoxR activation, we examined the contribution of the long C-terminal tail (18 aa from residue 158 to 175) that is specific to ScSoxR (Fig. III 19.a). The results indicated that this C-terminal tail is not responsible for the insensitivity of ScSoxR to paraquat or MDs (Fig. III 19.b). This coincides with what was previously reported (Sheplock *et al.*, 2013). However, the three key residues, whose mutation in PaSoxR to “enteric-type” residues increased its sensitivity toward paraquat and therefore were predicted to affect ScSoxR likewise (Sheplock *et al.*, 2013), did not behave as predicted in *S. coelicolor*. In our hands, all three mutations (V65I, P85L, L126R) did not change the selectivity profile, and the P85L and L126R mutations made ScSoxR less active even toward strong oxidant PMS (Fig. III 19.c,d). How phyla-specific conserved residues among SoxRs would contribute to the selectivity is an interesting and promising question. In addition, previously identified residues that affect redox potential in EcSoxR could serve as a good basis to search for their contribution in other SoxRs (Hidalgo *et al.*, 1997; Chander & Demple, 2004). Therefore, the residues and structural features that affect overall reactivity of SoxR toward RAC need be investigated in a more systematic way, preferably based on structural information.

The UV-VIS absorption spectrum of oxidized ScSoxR is similar but not identical to those of EcSoxR and PaSoxR (Wu *et al.*, 1995; Kobayashi & Tagawa, 2004). The EPR spectrum of reduced [2Fe-2S] in ScSoxR differs slightly from those of EcSoxR and PaSoxR (Fig. III-17). These discrepancies may reflect some subtle but significant differences in the environment of the

FeS cluster in each SoxR. Differential responsiveness toward not only the redox-active compounds but also NO may be the result of these differences in the cluster environments. NO activates EcSoxR by nitrosylating the [2Fe-2S] cluster of EcSoxR, forming protein-bound dinitrosyl-iron-dithiol adducts (Ding & Dimple, 2000). Considering its promiscuity, the Fe-S cluster of EcSoxR may be most exposed to solvent and/or its environment most flexible to accommodate any modifications of the cluster, compared with other SoxRs. The [2Fe-2S] cluster of ScSoxR may have a relatively restrictive environment that limits the accessibility and reactivity of the chemicals, and/or the sustainability of the oxidized/modified cluster to convey activation signal to the DNA-binding domain. In order to understand the features that determine the selectivity of reaction between [2Fe-2S] cluster of SoxRs and iron-reactive chemicals, careful systematic mutagenesis studies combined with physico-chemical analyses are in need.

Based on the relatively small number of regulated genes by PaSoxR or ScSoxR and the fact that they are activated by endogenous metabolites, it has been postulated that PaSoxR and ScSoxR are dedicated to responding to endogenous metabolites, in contrast to EcSoxR, which triggers a global stress response and responds to broad range of chemicals (Sheplock *et al.*, 2013). Our observation that PaSoxR resembles EcSoxR more closely than ScSoxR suggests that this functional classification is not that simple. The finding that even ScSoxR responds both to endogenous and exogenous natural metabolites as well as to xenobiotics implies that these non-enteric SoxRs are not specific systems for endogenous metabolites only. The differential range of activating chemicals may be an evolutionary outcome that reflects the ecological habitats of their host bacteria. The presence of other transcriptional factors in the same cell that can provide protective function in response to different array of chemicals might also shape the spectrum of chemicals to

which SoxR responds. *Streptomyces*, which primarily inhabit the soil and encode a large number of transcription factors that can divide labor, as exemplified by more than 700 transcriptional regulators present in *S. coelicolor* (Bentley *et al.*, 2002), could be best served by a SoxR of limited reactivity. On the other hand, *pseudomonads*, which are present rather ubiquitously from soil to human body, may be better off with a SoxR of broader reactivity. In order to understand the physiological role of non-enteric SoxR in further detail, genome-wide analysis of SoxR target genes and their functional analysis are needed in model organisms such as *S. coelicolor* as well as *P. aeruginosa*.

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